Study on the feasability of using microemulsions and PVA based gels in enzyme technology

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Ósnotur maður,
er með aldir kemur
það er bezt, að hann þegi.
Engi það veit,
að hann ekki kann,
nema hann mæli til margt;
veit-a maður,
hinn er vækti veit,
þótt hann mæli til margt.

Fróður sá þykist,
er fregna kann
og segja ið sama.
Eyvitu leyna
Megu ýta synir,
því er gengur um guma

The Stupid
Convening with strangers
His mouth he should keep shut!
Nobody'll notice
Him knowing nothing
If he can tame his tongue.
But that is the one thing
Where fools will fail:
To tame their tongue

He who can answer
And ask likewise
Is thought of as sensible
In company to conceal
For long time one's nature
Nobody is able.

Havamal (quoted after Briem 1968); English rendition by the author

Dedication

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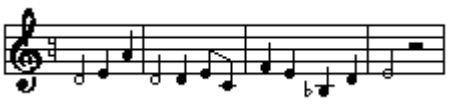

Abstract

Enzymes are interesting as catalysts due to their high selectivity and the ability to work under mild conditions. But many possible applications are hampered by their sensitivity, especially to nonaqueous solvents. It is therefore important to solve the problem of bringing enzymes into contact with water insoluble substrates while protecting them from detrimental solvent effects. Two important approaches are the immobilization on a carrier and the application of two-phase systems with a very large interface. In this work the suitability of water-in-oil microemulsions based on technical surfactants for this purpose is studied. Attempts were made to realize a (semi)continuous process for the enantioselective enzymatic reduction of prechiral ketones. This includes the pre-treatment of surfactants, the regeneration of the necessary cofactors (NAD(P)H) by another enzyme, the measurement of the reaction progress based on pH value shifts and the separation of the product containing oil phase from the aqueous reverse micellar phase by means of ultrafiltration. The different elements of the process are simulated based on existing and new models, and the simulation results are compared to experiments. Possible reasons for differences between simulation and experiment are evaluated by a qualitative study of the mixing characteristics of microemulsions and the deactivation of the enzymes in this environment. Fast enzyme deactivation due to contact with oil is observed during the period of microemulsion fomation. The study comes to the conclusion that under the current circumstances the microemulsion route faces too many obstacles in detail to justify a further pursuit at the present time.

A second part of this work deals with the suitability of crosslinked gels of poly(vinyl alcohol) for the immobilization of lipases. The effects of two different preparation methods on the microstructure are studied using scanning electron microscopy. Different application forms (foils, blocks, supported membranes, coatings) are compared qualitatively as to the effectiveness of the lipase immobilization. The obtained reactive membranes are also studied quantitatively using a filtration cell as reactor model. This study finds PVA based gels suitable for immobilization of lipase in membrane reactors.

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List of Abbreviations & Symbols

a_s	specific surface area	$[m^2/m^3]=[1/m]$
A	surface area	$[m^2]$
A_i	activity fraction	[-]
α	oil mass fraction in o/w mixtures	[wt.-%]
ADH	alcoholdehydrogenase	
c_i	concentration of component i	[mol/l]
cmc, cμc	critical micellar/microemulsion concentration	[mol/l]
c_{mic}	micellarly dissolved surfactant concentration	[mol/l]
CPCR	carbonyl reductase from *Candida parapsilosis*	
cPVA	crosslinked poly(vinyl alcohol)	
d	(hydrodynamic) diameter	[m]
d_{50}	median (pore) diameter	[m]
D	coefficient of diffusion	$[m^2/s]$
des, deg	deactivated/deactivation (indexed)	
E, E*, E_{deg}	enzyme (*=activated, deg=deactivated)	
ee	enantiomeric excess	[%]
η	dynamic viscosity	$[J/m^3*s]=[Pa*s]$
FDH	formate hydrogenase	
FT	freezing-thawing	
γ,γ	surfactant mass fraction	[wt.-%]
GDA	glutar(di)aldehyde	
HLADH	alcoholdehydrogenase from horse liver	
i	referring to component i (indexed)	
i	specific activity	μmol/min*g
I	total activity	μmol/min*g
I_0	initial activity	μmol/min*g
k_B	Boltzmann constant	[J/K]
k_i	(first order) reaction rate constant/coefficient	[1/s]
K_M	Michaelis-Menten constant	[mol/l]
m	mass	[kg]
$\dot{m}$	mass flow	[kg/s]
M	molar mass	[kg/mol]
$\mu_{p,m}$	median of pore/micelle size distribution	[m]
n_i	molar amount of component i	[mol]
NAD(P)+	ox.form of nicotinamide-adenine-dinucleotide (phosphate)	
$NAD(P)H_{(2)}$	red.form of nicotinamide-adenine-dinucleotide (phosphate)	
ox.	oxidized	
p	pressure	$[J/m^3]=[N/m^2]$
φ(x)	pore size distribution function	
Φ(x)	integrated φ(x) function [-∞;x] = permeation probability	
Ψ(x)	particle/micelle size distribution function	
perm	permeate (indexed)	
p-NPL	para-nitrophenyl laurate	
PVA	poly(vinyl alcohol)	
q	molar volume ratio	[-]
r	radius	[m]
r_i	reaction rate of component i	[mol/l*s]
r_c	radius of micelle core	[m]
r_t	thickness of surfactant layer around micelle	[m]

R	retention	[%]
r_{ADH}, r_{FDH}	reaction rate of ADH/FDH	[mol/l*s]
red.	reduced	
ret	retentate (indexed)	
RT	room temperature	
$\sigma_{p,m}$	standard deviation of pore/micelle size distribution	[m]
surf	surfactant (indexed)	
t	time	[s]
T	temperature	[K]
τ	residence time	[s]
tot	total (indexed)	
$V_{(0)}$	(initial) volume	[m^3]
$\dot{V}$	volume flow	[m^3/s]
w_0	total molar water/surfactant ratio	[mol/mol]=[-]
w_1	micellar molar water/surfactant ratio	[mol/mol]=[-]
yADH	alcoholdehydrogenase from yeast	

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0.General Introduction, Theme

Catalysis has become one of the central fields of chemical research and is now on equal basis with the synthesis of new compounds. Few chemical processes of economic importance do not employ catalysts of one kind or another [Festel 2004]. Many processes are actually built around the catalyst and whole industries have risen and fallen based on developments in the field of catalysis (the best known example being the production of sulphuric acid). Catalysts have been used millenia before even the most basic ideas about their effects developed, not to speak of the fact that life itself can be seen as a web of self-organizing catalytic processes. Even today our knowledge remains fragmentary, trial and error are still the daily bread of the researching scientist.

The chemical nature of catalysts is diverse to the extreme and ranges from the most simple (like the H^+ ion) to the most complex (currently: enzymes), from gas to solid, from single and simple compounds to intricate networks. They can facilitate seemingly trivial reactions (like the synthesis of ammonia from the elements) or trigger multi-step sequences leading to highly complex compounds (e.g. alkaloids or steroids) from simple building blocks.

One aspect that has become central is the ability of special catalysts to not just facilitate reactions but to do so in a highly selective way. With the right catalyst it is possible to selectively target specific positions in multifunctional molecules and to manipulate them without affecting other parts. It is also the key for the control of the stereochemical characteristics, essential for the synthesis of modern pharmaceuticals. In this area there is a rivalry between artificial catalysts, developed and synthesized from scratch, and the use of naturally occuring compounds taken or adapted from living systems. The advantage of the former is that they can be more easily adapted to different reaction conditions. Their structure is known and can be modified at will. As artificial compounds they are more easily obtained in pure form. Natural catalysts, taken from living systems (also known as biocatalysts with enzymes as the dominating class) on the other hand have to be taken as given. Modification and optimization are not (yet) standard options. Their usually fragile nature limits the conditions under which they can be used. Often it is even useful to leave them in their natural environment as in the case of whole cell catalysis (e.g. the use of living yeast instead of extracting the yADH). Although there are extremophile examples [Gomes 2004], they are not available for many economically interesting reactions. To find suitable biocatalysts and to obtain them in a sufficiently pure form is still an art and often depends on a good deal of good luck. What makes

enzymes important nonetheless is their still unmatched selectivity and specificity. They often allow for far milder conditions to be used than with artificial catalysts (Haber-Bosch vs. natural fixation of nitrogen being one of the most extreme examples).

This high selectivity comes at a cost. Many interesting compounds to be catalytically modified do not fall into the closer range of the natural substrate spectrum of the enzymes. Parts of the molcecule may be too bulky to fit easily into the active site or it may be insufficiently water soluble. Since the individual enzyme itself cannot be easily modified, it is necessary to check a selection of naturally occuring variants and to create conditions that both the biocatalyst and the substrate can share without or with only minor detrimental effects. These conditions have then to be developed into a working process that allows a reliable substrate conversion and extraction at justifiable costs. A general overwiev of the field can be found e.g. in [End 2004] or [Held 2000].

In principle enzymes are homogeneous catalysts, i.e. they are dissolved in the reaction medium and can move freely, reducing transport limitations and allowing for higher flexibility. Technical processes on the other hand are still dominated by heterogeneous catalysis with the catalytic component immobilized on a carrier. This allows for an easier handling and avoids the problem of having to separate the catalyst from the product stream (although there are interesting proposals to solve that problem [Haupt 2005]). But one has to buy these advantages with potential mass transport limitations and, as a common result, reduced selectivity. Enzymes often show a reduced activity when immobilized. On the other hand immobilized enzymes have often been found to be more stable than in the dissolved state, showing a longer half-life period [Shchipunov 2004] and greater tolerance to environmental factors (e.g. pH value, temperature, solvents), e.g. [Petzelbauer 2002].

The conventional approach is to either find an ideal carrier or to someheow stabilize the free enzyme, protecting it from denaturation, especially by non-aqueous solvents [Abian 2001]. Porous beads of organic [Svec 1999; Krajewska 2004], inorganic [Fontes 2002] or mixed [Chang 2005] materials and a wide range of encapsulation techniques [Prüße 2002; Gonzáles-Sáiz 2001] dominate the first field, non-conventional solvents [Eckstein 2004], solvent stabilizers [Goto 2005] and diverse liquid-liquid 2-phase systems [Doumèche 2002] the latter. An ideal process would be able to combine the advantages of both free and immobilized states while minimizing the adverse effects of either.

One possible approach, pioneered e.g. by Luisi [1985, 1988] or Larsson [1987], is the use of microemulsions, another that of membrane reactors [Rissom 1999; Liese 2002; Rios 2004], especially reactive membranes [El Masry 2000; Deng 2004; Magnan 2004].

Water-in-oil microemulsions keep the enzymes in an aqueous environment, partially protecting them from denaturation by organic solvent, while providing at the same time a huge interface, so that even substrates of very low water solubility can reach them easily from the continuous oil phase. Whether a microemulsion can have a stabilizing effect on enzymes other than lipases, as earlier studies indicate [Berger 1999], or are more likely to be detrimental [Melo 1998], is one of the main topics of this study. It will also, in continuation of the work of Schomäcker, Orlich et al., focus on the use of technical nonionic surfactants since these are both cheaper and less environmentally damaging than many alternatives in use before.

The themodynamic stability of the microemulsion, as opposed to conventional emulsions, does not require permanent vigorous stirring, thus removing a stress factor. In principle the surfactant-coated water droplets (in the nanometer range) can be separated from the oil phase by ultrafiltration, as if they were solid particles. This opens opportunities for a process with the enzymes immobilized in an almost free state in the reactor, while the oil phase containing the substrates and products can be exchanged continuously. Earlier studies [Schomäcker 1996, 1997; Orlich 1999] have shown that this is true in principle but highly dependent on the microemulsion parameters and the used membranes. This study will take a closer look on the practical applicability.

Many ecomomically interesting enzymatic reactions require the use of cofactors as providers of necessary redox equivalents (e.g. hydride carriers). Since these are expensive, they can't be used stoichiometrically but have to be regenerated [Kragl 1996]. Of the numerous known methods (electrochemically [Rehm 1998], chemically [Hilhorst 1983], enzymatically [Leonida 2001]), the use of another enzyme seems to show the greatest prospects. The resulting coenzyme cycle can be realized in living cells [Anderson 1998; Wandrey 2004] but also in the water droplets of a microemulsion. Several different enzymes have been proposed for this purpose [Vrtis 2002; Riebel 2003] but formate dehydrogenase [Neuhauser 1998; Tishkov 1999] seems to be the ideal candidate, since it yields CO_2 as "waste product". An extra separation step can therefore be avoided. This study will also test, whether the pH shift caused by the regeneration process can be used to easily measure the reaction progress because this would significantly simplify the on-line analytics.

It will then be attempted to combine all these elements into a continuous process and to compare simulations based on theoretical models of the same with the experimental results.

Additionally to the microemulsion route this study will take a closer look on the preparation of membranes, foils etc. from poly(vinyl alcohol) and the immobilization of enzymes on or in this material. It will be tested, whether the previously used freezing-thawing process [Djennad 2003] can be simplified, what effect this has on the microscopic structure and how it influences the enzyme immobilization and activity.

1.Theory

1. 1 The importance of being chiral

Many chemical compounds do exist in forms that relate to one another like image and mirror image, i.e. their chemical structure is identical but can not be brought into congruence. Because this is similar to the relation of the left and right hand, this penomenon is called chirality (from classical Greek χέιρ = hand). The compounds themselves are called enantiomers. Their basic physical properties like melting or boiling point are identical and the only difference is in their optical activity. Chiral compound pairs rotate the polarization plane of light passing through by a characteristic angle but in opposite direction. In 1:1 mixtures the effect is cancelled out and therefore no optical activity can be observed. Such mixtures are called racemates. Deviating mixtures are described by the ee value, the enantiomeric excess, defined as:

$$ee = \frac{c_{high} - c_{low}}{c_{high} + c_{low}} \qquad \text{(eq.1.1.1)}$$

For racemates the ee value is 0, for enantiomerically pure compounds it is 1.

While the basic physical properties of the pure enantiomers are identical the same is not necessarily the case for racemates due to specific interactions between the enantiomers. This shows the fundamental difference whether a chiral compound interacts with an achiral or another chiral compound.

If two enantiomers react each with an achiral compound, the resulting products will be again enantiomers, provided that the reaction did not destroy the chiral center.

If the enantiomers on the other hand react with other chiral compounds, there can be up to four products consisting of two pairs of enantiomers. The relation of those pairs is that of diastereomers that have different chemical and physical properties. Those can be separated by conventional means while the enantiomeric pairs themselves cannot.

The separation of enantiomeric pairs, called the resolution of racemates, requires symmetrically selective methods, e.g. the temporary conversion into diastereomers.

This will be discussed in more detail below.

Because the ambiguity increases exponentially with the number of chiral compounds involved, any complex three-dimensional system (like a living organism) needs to keep that aspect under tight control.

Biological functions are therefore to a large degree enantiomerically coded, i.e. they depend on the presence and action of a specific enantiomer. The opposite enantiomer will in most cases either have no effect or one different from the other. In harmless cases

this may simply result in a different taste (R-asparagine being sweet, S-asparagine being bitter for example) but the effects can be fatal too. The most notorious example is Thalidomide (Contergan®) (Fig. 1.1.1). While the R-configuration is a versatile drug (a non-addictive sedative also used in the treatment of leprosy and certain cancers), the S-configuration turned out to be a potent teratogene. Unfortunately, in the human organism a partial racemisation takes place, so female patients are only to be treated in combination with contraceptives [Bernstein 1999, Daemmrich 2002, Roth 2005]. The Contergan tragedy led to major changes of the laws and regulations governing the testing and approval of new drugs [Hasskarl 1979].

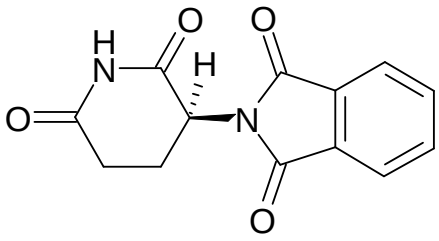


Fig. 1.1.1 Thalidomide (Contergan®)
The S-config. (imaged) is a potent teratogene
The R-config. is a versatile drug

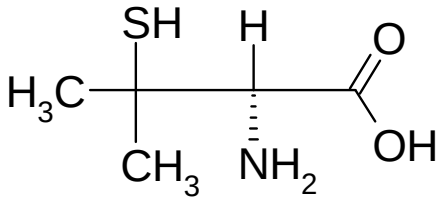


Fig. 1.1.2 Penicillamine
L-configuration: higly toxic
D-configuration (imaged): versatile drug

Occasionally it can be even the naturally occuring compound that has the noxious effect. L-penicillamine (Fig. 1.1.2) as derived from naturally occuring amino acids is a strong poison because the organism is unable to differentiate it from the related proteinogenous acids. It will therefore be used for the build-up of vital proteins inflicting on them a deviant structure thus rendering them inoperable. The "unnatural" D-variant on the other hand is effective against Morbus Crohn, other heavy metal poisoning (acting as a chelating agent), arthritis (exact mechanism yet unknown) and cystein kidney stones (by breaking disulphide bridges)[Römpp].

Medical regulations (e.g. the US Federal Food Drug and Cosmetic Act) of today therefore require the producer of a new pharmaceutical compound to either proof the harmlessness of the undesired enantiomer or to guarantee that the drug in question contains only the enantiomer responsible for the desired effect. Thus unnecessary side effects caused by the "wrong" enantiomer can be excluded or at least diminished. Racemic mixtures and the constituent enantiomers require separate application [FDA 1,2].

Data obtained [Festel 2004] show that of the top-100 drugs more than 50% are mono-enantiomeric already, corresponding to a turnover of ca. 100 billion US$ (state 2004).

60% of drugs in the later stages of development at that moment were chiral, and in 90% of those cases the synthesis is enantioselective from start on. This has the added

advantage to both cut the necessary amount of raw materials by half (driving down costs) and reduce the total dose to be given a patient.

1.2 Chiral synthesis and optical resolution of racemates

In order to selectively obtain one enantiomer, the correct chirality has to be either induced externally, introduced via natural chiral building blocks or achieved by separation of the enantiomers, i.e. the resolution of the racemate.

This work will deal for its main part with the external induction of chirality by certain chiral catalysts. Certain compounds have a prechiral character. They can be turned into chiral compounds with a single reaction that does not introduce a chiral group itself but transforms a preexisting achiral one into a chiral. Typically the prechiral compound possesses a single symmetry plane that allows to differentiate between a left (si) and a right (re) side. Dependent on from which side the attack occurs, different enantiomers are formed.

Should there be a second symmetry plane then the structure is not prechiral (Fig.1.2.1) because in the reaction product image and mirror image are identical.

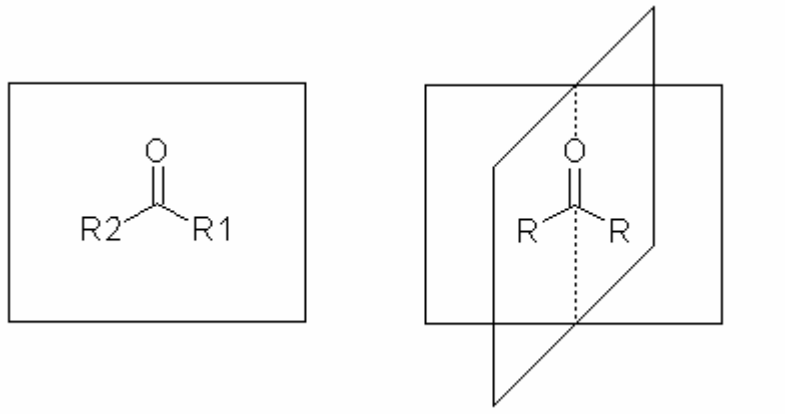


Fig.1.2.1 Asymetrically substituted carbonyl function (left) has a single symmetry plane and is prechiral. The symmetrically substituted carbonyl function with 2 symmetry planes (right) leads to achiral compounds

If the target atom is carbon, the prechiral structure is typically of a trigonal planar structure. Common prechiral functionalities are C-C double bonds or secondary carbonyl groups (e.g. ketones). Of industrial importance are e.g. the reduction of ketones to secondary alcohols or the reductive amination of carbonyl functions (synthesis of amino acids, amphetamine). Enzymatic catalysis has practically driven out the conventional chemical processes due to higher enantoselectivity, purer products and lower costs.

In the case of internal double bonds two stereo centers can be formed by addition of identical atoms to both adjectant atoms (Fig. 1.2.2) Depending on the type of addition (from the same side or opposite sides) those can cancel each other out and lead to so-called meso compounds (if the atoms on both sides of the double bond were substituted symmetrically). The standard example for this phenomenon is tartaric acid.

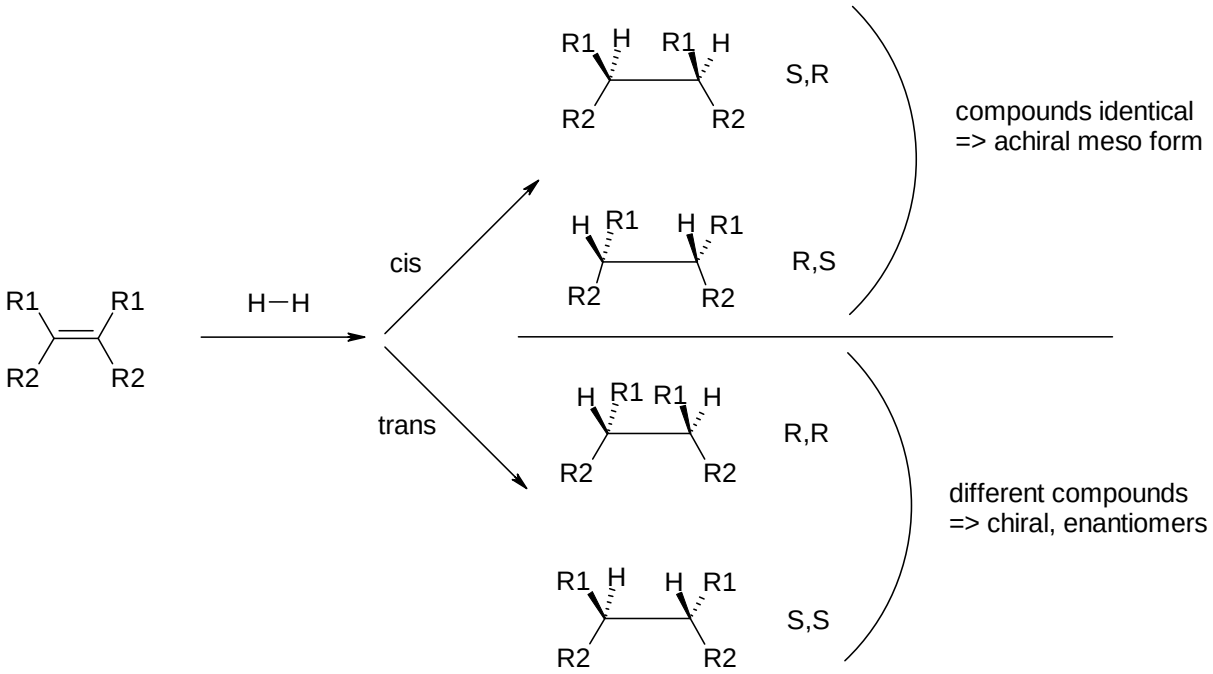


Fig 1.2.2 Addition to internal double bond leading to chiral or achiral compounds depending on cis or trans attack. Configurations under assumption that $R_1 > R_2 > CHR_1R_2$

The enantioselective hydrogenation is currently achieved almost exclusively by means of noble metal complexes with chiral "asymmetric" ligands. Enzymatic processes have yet to establish a position in that market.

If direct chiral synthesis is not achievable, at some point a resolution of the racemic mixture is necessary. There are basically two methods, static and dynamic. The static method consists of a reversible reaction with an available cheap chiral compound usually from the natural "chiral pool", e.g. an amino acid. The two products (salts in the most simple case) are diastereomers and therefore differ in their physical characteristics. They can then be separated by suitable methods and the reaction reversed, yielding the pure enantiomer.

The dynamic method, dominated by enzymatic processes, utilizes the fact that a suitable chiral catalyst will convert enantiomers at different rates or even exclusively one of them.

In order to resolve a racemate of enantiomeric alcohols or acids, the mixture is first esterified using a cheap reaction partner like acetic acid or methanol. The ester is then

partially saponified by a suitable lipase. Because the enzyme is biased towards one enantiomer, the product mixture will be enriched in the one enantiomer while the other mostly remains in ester form. After conventional separation of these compounds the process can be repeated until the desired enantiomeric excess is reached. This can be seen as analogue to the rectification of a mixture of compounds of different volatility.
The disadvantage of this method is that the maximum conversion is 50% (100% of one enantiomer, 0% of the other), usually less. This can be increased, if the remaining mixture can be easily racemised again. This is the only way to achieve a yield exceeding 50%.

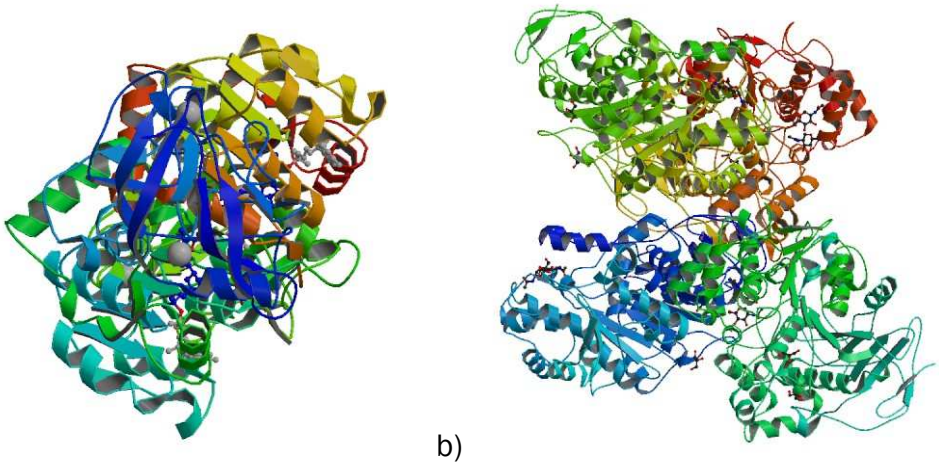
1.3 Enzymes and Coenzymes

Enzymes are catalysts from living organisms, therefore called biocatalysts. They are involved in every vital process (in both senses of the word) and exceed both in variety and performance anything hitherto available in conventional "lab" chemistry. While many artificial catalysts require rather harsh working conditions (pressure, pH value, temperature), biocatalysts, with few exceptions, evolved to achieve their optimum performance at very mild conditions. Unfortunately they are also limited to those because they consist of components sensitive to extremes of temperature or pH far from neutral values. Under optimum conditions they are highly selective but are also very "picky" concerning their substrates. Conventional catalysts allow a remarkable degree of fine-tuning by choice of support materials and ligands. Traditional catalyst research is therefore usually gradual. After finding (by screening) a basic compound able to catalyze a certain reaction type, the conditions are varied in order to increase the selectivity and yield.
In biocatalytisis the screening is still an all or nothing process. For certain reactions a catalyst has yet to be found (e.g. Diels-Alder) and there is no guarantee that any exists in nature. For other reactions (e.g. esterification/saponification) there is an abundance. Even if our understanding of the catalytic mechanism is at times higher than with "traditional" catalysts, we can't manipulate them yet the same way we can conventional catalysts. Even worse, the biocatalyst is optimized for its natural conditions and may behave rather different under artifical conditions of a chemical reactor, while synthetic catalysts are developed for those conditions. Biocatalysts used today are either taken from nature or modified from existing natural "models". Our knowledge is still far from giving us the the ability to design an enzyme from scratch or to deviate too far from the basic building principles as found in nature. Although it it is technically possible to synthesize enzymes abiotically by means of solid-phase technique as pioneered by

Merrifield [Kresge 2006] and chemical ligation [Dawson 1994], allowing the free use of building blocks not naturally occuring, it is of no technical significance (the same way that the full synthesis of morphine [Gates 1952/6] was a scientific achievement of no relevance for the drug market). The basis for technical biocatalysis is therefore still the living organism as the producer of enzymes. There are today two basic methods to modify such existing enzymes: 1. recombinant DNA technique, i.e. changing the genetic blueprint the producing organism uses for the biosynthesis [Pasternak]; 2. Chemical modification of the enzyme by conventional means, e.g. alkylation of the secondary functionalities of the constituting amino acids on the outer surface. While the latter aims at the interaction of enzyme and solvent (comparable to the choice of ligands in homogeneous metal catalysis), the former can also be used to influence the catalytic function itself, the spectrum of possible substrates etc.

The extraction and purification of enzymes is a difficult process and often requires the destruction of the producing organism (except in the cases of extracellular enzymes like many lipases). Natural enzymes consist of chain(s) of amino acids that by a process of self-organisation shape into a three-dimensional structure (examples can be seen in Fig.1.3.1).

Some of the monomers in the chain are mainly 'fillers' and can vary between different strands, while others are essential because they actively determine the geometrical shape and/or the interaction with suspension media. The solubility in different media and the possibility of covalent immobilization are highly dependent on the secondary funtionalities of the amino acids at the periphery of the enzyme structure.



a) b)

Fig.1.3.1: 3-dimensional models of enzyme structures:
a) alcohol dehydrogenase from horse liver complexed with NAD+ and 2,3-difluorobenzyl alcohol
b) lipase from *Candida rugosa* (closed state). [Protein Data Bank rcsb.org]

At the core of the structure the active center of the enzyme is located, where the actual catalysis takes place. With some enzymes, e.g. lipases, the spatial positioning of certain amino acids is sufficient to provide the catalytic effect. In most cases a chemically different entity has to be added in order to achieve the desired result. This other entity can be either built in and be inseparable from the enzyme or it can be separate and interact with it like a substrate. In special cases it can be linked to the structure but still essentially can act freely. In the former case this is known as the prosthetic group and can consist of one or more metal ions (almost exclusively transition metals) or an organic molecule. In the latter case terms used are cofactor, coenzyme or cosubstrate depending on the context. The term cosubstrate is disputed though because it ignores the difference between enzyme independent compounds that are e.g. just chemically linked by the enzyme (like alcohol and acid in esterification) and those molecules that only act as intermediate and are regenerated externally (but are essentially an integral part of the catalytic process). Their function can be for example to activate the main substrate by reacting with it and being eliminated after the biocatalyzed main reaction.

The function of these cofactors vary. They can allow the actual substrate to bind to the active center in the first place, they can undergo temporary changes thereby stabilizing the transition state (e.g. delocalizing charges), they can provide or receive atoms or groups in transfer reactions ('carriers'), play the second partner in a redox couple and (at least theoretically) determine the regio- or enantio-selective outcome.

In some cases the exact relation of enzyme and coenzyme(s) is still not fully understood. Metoxatin(PQQ) and related quinoproteins are the prime examples here [Zatman 1967, Matsushita 1997, Kasahara 2003]. An enzyme may accept or even require more than one, those may only work in connection with other compounds etc.[Kvamme 1968]. This is part of the reason, why some promising enzymes seem not to work outside their natural environment.

The coenzymes dealt with in this work belong to the carrier/redox couple variety. They transfer hydrogen ions thereby providing redox equivalents. The most common coenzymes follow a standard architecture. The active group is linked by a sugar (open or ring) or an amino acid, an oligophosphate and another sugar (always ring) to a nucleobase (default: adenine) as can be seen in Fig. 1.3.2.

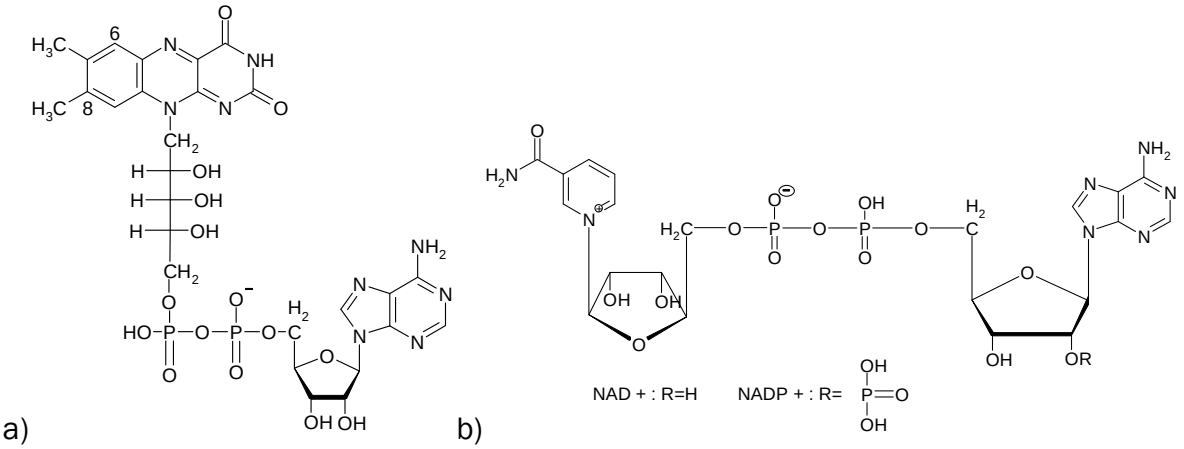


Fig.1.3.2: Examples of Coenzymes a) FAD b) NAD(P)+/NAD(P)H
While NAD(P)H only occurs in the free form naturally, FAD can also be found attached to the enzyme either in the 6 position or over the methyl group in 8 position

The connections between those parts are the Achilles heels of the molecule and mainly responsible for their short lifetime and low total turnover numbers. The phospate group in particular makes the coenzymes sensitive towards phosphate buffers [Rehm 1998], i.e. they decay at a higher rate in their presence. Unfortunately the removal of these seemingly redundant parts usually results in the coenzyme not being able to bind to the enzyme any longer. NADH/NADPH demonstrates this in yet another way. The small difference of an extra phospate group on the ribose ring connected to adenine makes NADPH much more sensitive and significantly reduces the half-life time in both native and lab conditions. Oxidoreductases (like ADH or FDH) of identical function accept with few exceptions either the one or the other but not both as coenzyme.

To find less sensitive replacements is a topic of research [Lo 2002] but a perfect solution seems not to have been found yet. The possible turnover numbers for the active group itself are much higher [Rehm 1998], i.e. it can undergo far more cycles without being deactivated by a side reaction than the rest of the molecule. So there is a true incentive to find an inert structure that can replace the sugar-phosphate-sugar bridge and is recognized by the enzyme. For more background see: [Bucholz 1997]

1.4 Reaction mechanism of alcoholdehydrogenases

While for lipases the enzymatic catalytic reaction mechanism has been determined in detail in the 1990-ies [Tilbeurgh 1993; Schmid 1998], the same cannot be said about alcoholdehydrogenases.

It is known that ADH enzymes employ (usually two) Zn^{2+} ions in their active site [Bränden 1975] but the exact function of at least the second ion is under dispute [de Souza Pereira 1998; same author also discusses general mechanism of different ADH].

Most known ADH enzymes share two certain features:

1. They work best on methyl ketones. The reaction rate drops dramatically, if the methyl group is replaced by a significantly larger (i.e. more bulky) residue. The larger rest can be varied much more widely with only a gradual drop in reaction rate.
2. The enantioselectivity of the isolated enzymes is usually very high (approaching 100%).

This is commonly explained with the simple 2-pocket model (Fig.1.4.1). At the active site there are two pockets of different size. One has room for only a methyl group (or a methyl group with hydrogen replaced with other atoms of limited size), the other, larger one (also named the hydrophobic channel) [de Souza Pereira 1998], can take up a much bigger residue and also influences the substrate specifity through the adjacent amino acids. The asymmetric (pre-chiral) ketone is thus fixed in a defined configuration in relation to the body of the enzyme. The co-enzyme will therefore always approach the C=O group to be reduced from the same side. In the majority of known ADH enzymes this is the *re* side, resulting in the formation of s-alcohols [Peters 1998]. Because the two active hydrogen atoms in NAD(P)H are chemically identical, the reaction should only depend on the relative orientation of the co-enzyme molecule (=> rate identical for enzyme and iso-enzyme [not occuring in nature]).

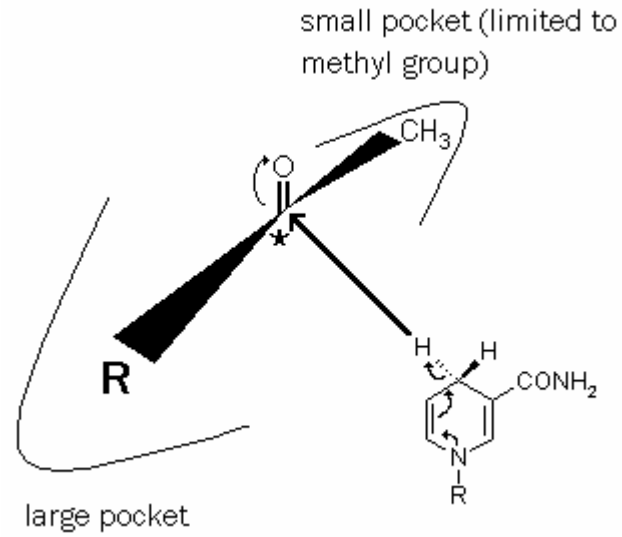


Fig 1.4.1: 2-pocket model of the enzymatic reduction of ketones by ADH enzymes explaining the enantioselectivity of the nucleophilic hydrogen transfer

By change from the neutral quinoid form into the positively charged benzoid form of the nicotinamide ring, one active hydrogen is released as a hydride that acts as a nucleophile at the pre-chiral carbon of the carbonyl group of the ketone. Thus the ketone is converted

enantioselectively into an alcoholate that will abstract a H^+ ion from water in order to be neutralized. Whether the latter step occurs before or after the alcohol is released from the active enzyme site, seems not to have been revealed so far.
The reaction therefore consists formally of the addition of a neutral hydrogen molecule to the C=O double bond of the ketone. Only one of the hydrogen atoms originates from the co-enyzme, the other is taken from solvent water thus increasing the pH value of the system. Further literature: [Fersht 1999, Schürer 2006]

1.5 Reaction mechanism of lipases

Lipases catalyze the esterification/saponification reaction involving primarily long-chain (fatty) acids. The mechanism, depicted in Fig 1.5.1, in all known cases involves the catalytic triad consisting of serine, histidine and aspartate (can be replaced with glutamate) that are linked by hydrogen bonds. The serine residue can act as a nucleophile on the ester carbon because the histidine is able to take over the hydrogen from the serin hydroxy group (bond switch). The hydrogen is then transferred to the forming alcoholate.

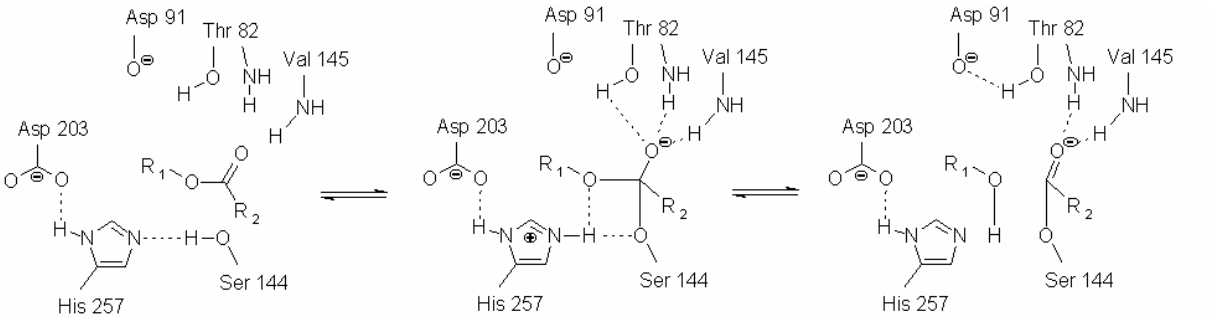


Fig.1.5.1 : Catalytic triad in lipases. The mechanism is universal, the depicted example is from *Rhizopus oryzae* (figure redrawn from [Schmid 1998])

The intermediate oxyanion of the carbonyl group is stabilized in the tetrahedric transition state by the so-called oxyanion hole formed by two amino and one hydroxyl functionality.
The necessary steric conformation has to be triggered by a hydrophile/hydrophobe interface and the lipophile residue of the fatty acid. The interface induces a conformational change of a "lid" covering the active site allowing the substrate to enter [Tilbeurgh 1993]. The presence of the lipophilic residue then causes a secondary change that allows the stabilization of the transition state. This explains why lipases especially work on long-chain compounds and show much lower activity, if the chain length is

reduced. The lid structure is the main classification feature differentiating lipases from esterases.

1.6 Immobilization of Enzymes

The main part of this work will deal with the immobilization of ADH enzymes in microemulsion droplets and to a lesser extend with lipase fixation on membranes. This chapter will focus on methods as established in literature.

The term "immobilization", taken literally, has a wide meaning. Since there seems to be no universally accepted definition, it will, for the pupose of this study, be defined as encompassing all methods of keeping a selected compound (usually a catalyst) within the confines of a defined non-closed system, i.e. a system that other compounds can enter and leave freely. This can be accomplished by either making the borders of the system selectively (im)permeable or by preventing the compound to come in contact with the borders. The first method utilizes some kind of filter, e.g. a membrane, the second requires a fixation of the compound to a carrier. An intermediate between those extremes can consist of a coupling of the individual compound molecule with a bulky residue ("ball and chain") or with other molecules of the same compound (analogous to a chain gang). In this way the size is increased to a degree that does not allow the compound to pass through the filter but still allows the free movement within the system. Thus the whole area from homogeneous to heterogeneous catalysis is covered. Table 1.6.1 gives a general overview.

Table 1.6.1 Classification of immobilization methods (after [Tischer, Wedekind 1999]).

Immobilized Enzymes		
Attachment to (pre)fabricated carriers	**Crosslinking**	**Encapsulation / Inclusion**
covalent binding	direct crosslinking	**membrane devices**
ionic binding	crystallization + crosslinking	microcapsules
adsorptive binding	co-crosslinking	**liposomes/reversed micelles**
metal binding	copolymerisation after chemical modification	organic solvents
(bio)affinity binding		

Each method has its distinct advantages and disadvantages to be discussed below. There is no universal option of choice but the method to be employed has to be selected individually for each enzyme. This is still more of an art than a science and trial and error still dominate the applied research.

In conventional catalysis the retention of the catalytic compound is one of the main topics because the metals used are often very expensive (e.g. Pt, Rh) or harmful (e.g. Hg, Os). In enzymatic systems keeping the catalyst in an active state plays a far larger role due to their sensitive nature, while their size allows a relatively simple separation by e.g. ultrafiltration. Often a compromise between activity and stability/longevity has to be made because many enzymes are less active but more stable when immobilized [Tischer 1999]. In the case of the use of a carrier there is the additional problem of leaching. Often a firm (leach-proof) fixation of the enzyme on the surface of the carrier material results in severely curtailed activity, while a loosely fixed but active enzyme is washed out more easily. If the enzyme requires more than a single substrate (as is the case with redox enzymes like the ADH type), another partial or complete immobilization of the co-substrate may be necessary. Free immobilization is the preferred method here because the selective fixation of enzyme and co-enzyme/substrate within range of each other has not yet been mastered to a sufficient degree, though attempts have been made to link a coenzyme to the enzyme via a hydrocarbon chain [Obón 1998].

All processes employing immobilization are hampered to a certain degree by transport limitations, i.e. the immobilized catalyst is less accessible for the substrates, and inhibition effects can be intensified by this too. Any fixation of enzymes at a carrier has to address a number of problems related to their nature.

The 3-dimensional structure of an enzyme is not a rigid one and quite sensitive to distortion by external forces. The amino acid sequence (primary structure) leads to relatively stable spatial organization of substructures (secondary st.). Those interact through several different weak forces supported in places by disulphide bridges to form a rather more flexible macrostructure (tertiary st.). On occasion (as is the case with ADH enzymes), several of those can interact non-colvalently to form an oligo-unit (quartary st.). The activity of a protein as a catalyst is facilitated by highly specific conformational changes of mainly the tertiary structure. The intermolecular forces exerted by a specific substrate induce a counteraction from the protein, changing its structure accordingly. This is called the induced fit. If other external forces influence the enzyme structure, this induced fit may be impeded or blocked completely (inhibition).

If the enzyme is immobilized on a carrier, the flexibility is usually reduced. This can be beneficial, if it prevents strong distortions but still allows the induced fit. In this case the performance can be even enhanced in environments that are usually detrimental. In most cases, unfortunately, the negative effects of distortion by fixation outweigh these potential advantages and the increased stability is achieved at the price of diminished

activity only.

Knowledge about the detailed enzyme structure can help to roughly predict the extent of positive and negative effects of different fixation methods but much is still left to trial and error. The knowledge about the amino acid sequence can for example influence the choice of crosslinker between carrier material and protein. Functional groups that exist mainly at the periphery but are rare near the active site would be preferred. Those that would make the active site the main linking target would be avoided. The number of links between carrier and protein has to be balanced too, weighing up the positive effects of firm fixation (stable, leach-proof) against the negative of reduced activity by distortion.

A useful anti-distortion countermeasure proposed by for example [Chae 2000] is to toggle the enzyme during the fixation process, i.e. to (temporarily) force it into the active conformation. This can be achieved by having a high ($>K_M$) concentration of intended substrate present. This way most enzyme molecules will be in the induced fit conformation when immobilized, reducing the effect of random distortion. This is of course limited by possible reactions of substrate with activated carrier or crosslinker.

A promising principle is the imitation of the natural environment of an enzyme, the cell, while omitting the known disadvantages (metabolic needs, rivaling chemical processes etc.). That means the construction a micro-container for the enzyme(s), possible cofactors and usually a solvent that is tolerated by the biocatalyst. The boundary of that container has to be penetrable for the substrates and the products but not for the contents. The size of that container has to be a compromise between the enzyme's spatial needs, the need for a high relative surface area (in order to reduce transport limitations) and the retainability with standard filters. In whole cell biocatalysis hollow polymer spheres are in use. For the much smaller enzymes the term container is more flexible and does not necessarily imply a rigid structure, nor even a hollow one. Lipases have for example been embedded in dense polymer globules in the sub-millimeter range that still allow the enzyme to be reached by substrates through diffusion but nonetheless protect it from the hostile environment. Stretching the term even further, dendrimers that can adsorb and desorb enzymes depending on the ionic strength of the medium are discussed as reusable containers.

Closest to the natural cell are artificial vesicular and micellar structures that form a liquid barrier around the enzymes. A vesicle is a closed double layer membrane of amphiphilic molecules that can widely vary in size, the inner and outer side are equal in polarity. A micelle has a single shell of amphiphilic compounds only and different polarity at the in- and outside. If the inside is the polar one, the term used is inverse or reversed micelle.

The size of micelles is far more pre-determined by the specific conditions than that of vesicles and dependent on the environmental characteristics, like the amphiphile used, the nature of the continuous outer phase, the nature and amount of the dispersed inner phase. The size of a micelle can thus be calculated, if environmental and equilibrium data are given. These micelles are also dynamic structures and are in permanent equilibrium with each other and monomerically dissolved amphiphile in the continuous phase. The study of a subset of these micellar structures, microemulsions, and their use for the immobilization of enzymes is a main topic of this study.

1.7 Surfactants, Emulsions and Micoemulsions

It is proverbial that oil and water don't mix. Apart from a minute mutual solubility a forced mixture of water and non-polar hydrocarbons will separate into two distinct phases once the coercive external force is removed. Driven by surface tension droplets will spontaneously coalesce, and the difference in density results in the vertical separation of the growing domains of both components. A very fine dispersion, especially if minute impurities are present, may procrastinate the completion of this process for a few hours but the bulk separation takes place very quickly. In order to delay this, ideally indefinitely, the coalescence has to be impeded through the use of a solutizer. Chemical compounds containing polar (hydrophilic) and apolar (lipophilic) segments, known as heads and tails, can act as mediators, aggregating at the phase boundaries. There are 4 major types of these *surfactants*: anionic, kationic, nonionic and polyelectrolytic.

Table 1.7.1: Examples of anionic surfactants

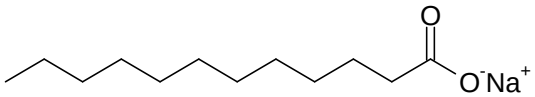
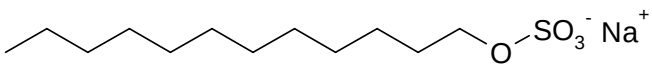
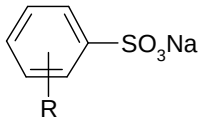
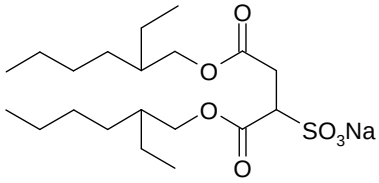
Anionic		
Type	Example	Name/Brand
Soaps		
Fatty alcohol sulfates		SDS
Alkylbenzene-sulfonates		
Sulfosuccinates		AOT

Table 1.7.2: Examples of cationic surfactants

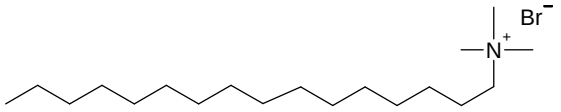
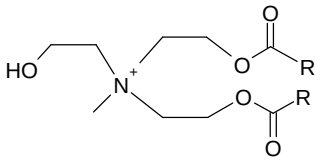
Cationic		
Type	Example	Name/Brand
Quaternary ammonium salts		CTAB
Esterquats		

Table 1.7.3 Examples of nonionic surfactants

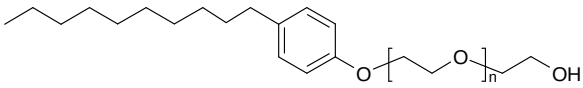
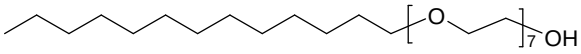
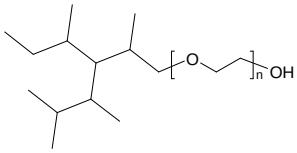
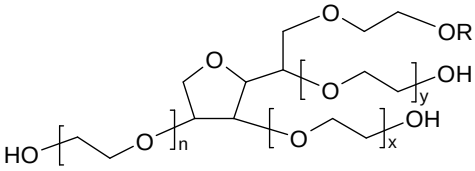
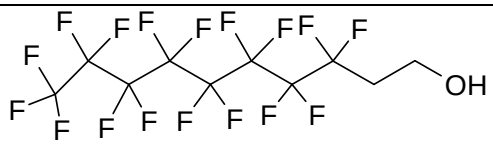
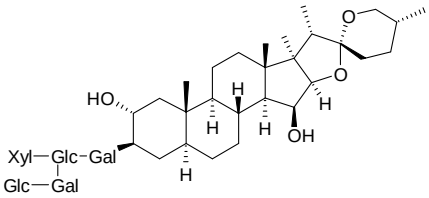
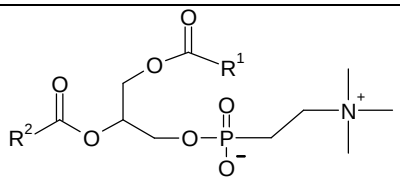
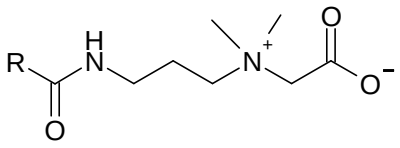
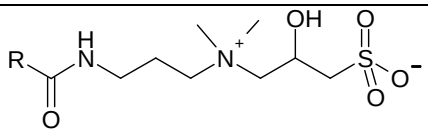
Nonionic		
Type	Example	Name/Brand
Alkylphenol polyglycol ethers		Igepal
Ethoxylated fatty alcohols		ENA-TDE7
Ethoxylated synthetic isoalcohols		
Sorbitane esters		Polysorbates Tween
Perfluorinated		FTOH 8:2
Natural Saponines		Digitonin

Table 1.7.4 Examples of amphoteric surfactants

Amphoteric		
Type	Example	Name/Brand
Natural		Lecithine
(Synthetic) Betaines		
(Synthetic) Sultaines		

First written records (cuneiform tablets) on the deliberate production of surfactants date from about 2500 BC and describe the treatment of natural oils (e.g. fat, tallow, lard) with woodash (containing potash or soda) thus producing soaps. Extracts from certain plants (e.g. soaproot) were also used directly. Those extracts contained not soaps in the chemical sense but saponines, natural (mostly) nonionic surfactants with an oligo-glycoside head and a steroidal tail (making many of them also potent poisons). Up to the Middle Ages these surfactants were mainly used not for body hygiene but for textile treatment and cosmetics. Fully or partially synthetic nonionic surfactants, mainly poly-glycol ethers) were introduced on a large scale in the 20th century and challenge the dominant position of the anionics. Both account for more than 90% of the total production while kationics are at about 7% and amphoteric/polylectrolytic at just 1% [Römpp].

The use of surfactants goes far beyond mere detergents and foaming agents. They can be found in all areas of life, from stabilizing the foam on beer (using algae extracts), preventing water from crystallizing in ice cream, reducing flow resistence in tubes to the gelation of incendiary fluids (Napalm, that also sticks to the skin as a result of the soapy ingredients).

The total amount of surfactant consumption is higher than 5 Mio tons per year and still increasing. In small amounts the main effect of surfactants is to slow down the coalescence of droplets through repulsive interaction between the polar headgroups, especially in case of ionic compounds (e.g soaps). Surfactant molecules accumulate at phase boundaries. The detergent effect results from the tail section connecting to

hydrophobic surfaces causing the heads to form a polar surface around the hydrophobic domains. Thus the solubilisation of these domains (e.g. water-insoluble dirt or drops of fat) is facilitated allowing them to be washed away. The solubilisation of hydrophobic liquid substances by surfactants is called an emulsion.

Conventional emulsions are at best kinetically stable but are not in thermodynamic equilibrium. Because the domains enclosed by the surfactant are larger than the wave-length of visible light, multiple scattering results in the emulsions being turbid or even opaque. The best known emulsion of this type is milk with droplets of fat stabilized by protein chains in a continuous water phase.

Emulsions can be classified as oil-in-water (o/w), water-in-oil (w/o or inverse) or bicontinuous systems. In the first two cases the dispersed compound forms segregated entities (e.g. spherical, globular, wormlike structures) in the continuous phase, in the last case the inner structure resembles a sponge. In any case the surfactant molecules are oriented in a way that the lipophilic/hdyrophobic tail points towards the oil domain and the hydrophilic/lipophobic head towards the water domain. Surfactant aggregates can themselves aggregate to form secondary structures, if the concentration is high enough. Liquid crystal phases may be formed that way.

The aggregation of surfactant molecules at the interface (or surface) is the result of the driving force towards minimizing energy. Water molecules around hydrophobic entities are forced into a cage-like structure because they can't form hydrogen bonds into that direction. The removal of the hydrophobic entity allows the breakup of this cage leading to a favorable increase of entropy. Likewise the removal of a polar compound from the oil phase allows the favorable solubilisation by water. The nonpolar attractive forces between lipophilic compounds favors their movement towards the oil phase. The orientation of surfactant molecules at the interface with head in water and tail in oil acts as a buffer reducing the higher energy of water and oil molecules at the interface.

In order to better understand the mechanism of surfactant-solvent interactions it is useful to have a look at the binary case, the dissolution of the surfactant in the solvent. In a binary solution of surfactant in either water or oil the above mentioned molecular forces trigger another behaviour. Above a certain concentration, mainly dependent on temperature, surfactant molecules aggregate to form microstructures analogue to those in an emulsion. At moderate concentrations these aggegations are spherical or globular and known as micelles (lat.: mica = little grain). The critical concentration above which additional surfactant molecules are used for the formation of micelles is therefore known as the cmc or cμc (critical micelle/microemulsion concentration).

If the fraction of surfactant in the ternary mixture of water,oil and surfactant is increased and/or (in case of ionic surfactants) a short-chain alcohol is added, a significant change can occur, marked by a sudden clear-up. This behaviour, first described in [Schulman 1943], is now known to be a true phase transition towards a themodynamically stable equilibrium state. This state is termed (since 1959) a microemulsion. Topologically similar to conventional emulsions, the classification quoted above can be applied here too. The microstructures are usually much smaller though. Dispersed droplets do not coalesce irreversibly but separate again. As a general rule the size distribution is negligible and the size is governed mainly by the molar ratio of dispersed compound to surfactant and the steric structure of the latter.

For a ternary or pseudo-ternary (if a cosurfactant is involved) mixture the appropriate graphic representation is the phase triangle. Because the temperature is also an important variable, the triangle is extended to a phase prism (Fig.1.7.1). 2-Dimensional diagrams are therefore sections through this prism.

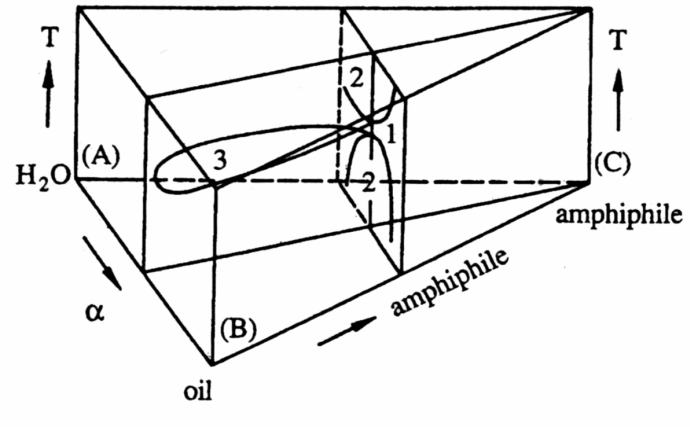


Fig 1.7.1. Schematic phase prism for a ternary mixture of oil/water/surfactant [Lade 1999]

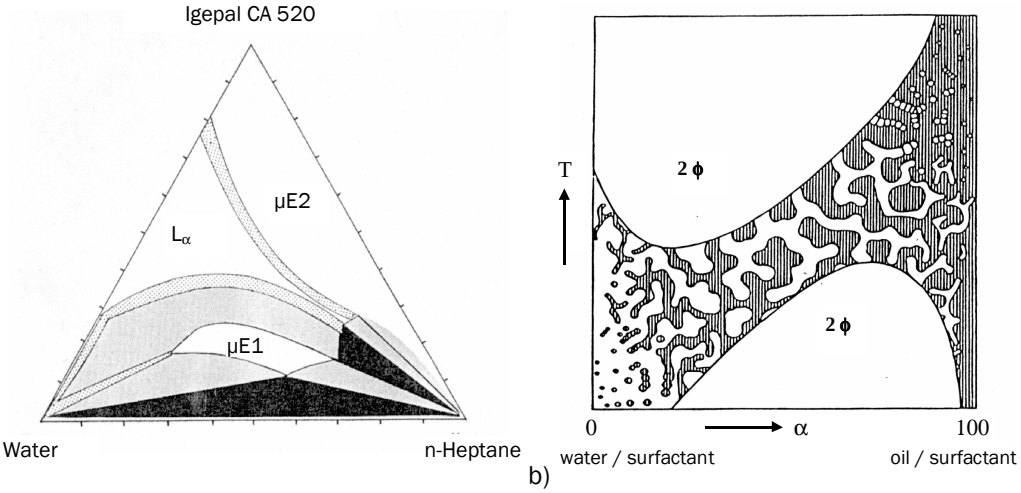


Fig 1.7.2 Sections through the phase prism
a) Ternary mixture of water/heptane/Igepal CA520 [Grätz 1998]; b) 1-Phase channel; left side: water-in-oil, right side: oil-in-water, center: bicontinuous [Schomäcker 1992]

Most common are sections parallel to the basis, i.e. phase triangles at constant temperature, and those parallel to the water/oil base line. In the latter case a rectangle is constructed with the temperature as the y axis and the relative oil/water ratio as the x axis (the fraction of surfactant is constant). Typical examples can be seen in Fig.1.7.2b.
Microemulsions and related systems are customary described by a set of three variables:

$$\alpha = \frac{m_{Oil}}{m_{Oil} + m_{Water}} \qquad \text{(eq.1.7.1)}$$

i.e. the weight fraction of oil in the water/oil mixture (surfactant fraction constant),

$$\gamma = \frac{m_{Surfac\tan t}}{m_{Surfac\tan t} + m_{Oil} + m_{Water}} \qquad \text{(eq. 1.7.2)}$$

i.e. the weight fraction of surfactant in the ternary mixture,

$$w_0 = \frac{n_{Water}}{n_{Surfac\tan t}} \qquad \text{(eq. 1.7.3)}$$

i.e. the molar ratio of water and surfactant.
α indicates, whether an oil in water (low α), a water in oil (high α) or a bicontinuous system (medium α) can be expected. γ is an indicator of the total size of the oil/water interface. w_0 gives information on the volume/surface ratio of micelles and thereby allows to estimate their size. Strictly spoken the cμc has to be subtracted before, and it is the resulting w_1 value that yields the size for the micelle:

$$w_1 = \frac{n_{Water}}{n_{Surfactnt} - n_{Surfac\tan t,c\mu c}} \qquad \text{(eq. 1.7.4).}$$

The relation of the water core radius r_c and the micellar water/surfactant ratio w_1 is:

$$r_c = \frac{r_t}{\sqrt[3]{\frac{q}{w_1} + 1} - 1} \qquad \text{(eq. 1.7.5)}$$

with the molar volume ratio

$$q = \frac{M_t * \rho_{H_2O}}{M_{H_2O} * \rho_t} \qquad \text{(eq. 1.7.6).}$$

More background: [Stache 1981/1990], [Brezesinski 1993], [Dörfler 1994], [Lade 1999], [Ueda 1980], [Sottmann 2002], [Burauer 1999]

1.8 Model for filtration with porous membranes

Under ideal circumstances a porous membrane would possess pores of a single size. The retention of particles would solely depend on their diameter. The distinction would

therefore be binary: total permeation or total rejection for any given particle size. In reality this is not the case, and the classification of membranes uses the cut-off number for the description of the separation factor. The cut-off can be defined over a length (sieve constant) or a weight (molecular cut-off). The standard definition is the retention of 90% of particles exceeding the cut-off number.

Several factors contribute to this "fuzzy" border. The ideal model postulates spherical hard particles, straight cylindrical pores and absence of interaction (apart from geometrical) between both. In reality none of these conditions are fulfilled exactly. Hydrophilic/hydrophobic interaction can be of major importance, and particles are often easily deformable. The latter is largely responsible for the pressure dependence of filtration performance.

For simulation purposes the modelling will be restricted to the size distribution. On the membrane side the distribution of pore sizes covers the main area of factors contributing to the distribution of separation. On the particle side the size distribution has to act as a stand-in for the above mentioned host of influencing factors, i.e. even for a monodispersed solution a certain size distribution could be assumed to account for factors like deformability that are otherwise difficult to tackle.

The model makes the following assumptions:

1) Pore sizes (p) follow a Gaussian distribution
2) Particle sizes (micelles, m) follow a Gaussian distribution
3) A single particle is rejected if it hits a pore with a smaller diameter than its own
4) Particles hit pores statistically according to the given size distributions

From these conditions 4 parameters are obtained describing the filtration behaviour. Those are the median of the size distributions μ_p, μ_m and the corresponding standard deviations σ_p, σ_m.

$$\phi(x) = \frac{1}{\sigma_p \sqrt{2*\pi}} * e^{-\frac{(x-\mu_p)^2}{2*\sigma_p{}^2}}$$ Pore size distribution (eq.1.8.1)

$$\psi(x) = \frac{1}{\sigma_m \sqrt{2*\pi}} * e^{-\frac{(x-\mu_m)^2}{2*\sigma_m{}^2}}$$ Particle/Micelle size distribution (eq.1.8.2)

The Gaussian distribution has by definition the area 1. The probability Φ(x) of a particle with the size $x=d_m$ to be rejected is the definite integral of $-\infty$ to x of the pore distribution function.

$$\Phi(x) = \int_{-\infty}^{x} \phi(t)dt$$ (eq.1.8.3)

The permeation probability is accordingly $1-\Phi(x)$.

For practical reasons the integration interval can be limited because the interval [-3 σ; +3 σ] already covers 99,7% (proposal: limits -5 σ or 0 to +5 σ).

For a monodispersed solution this is also the total retention. For a solution with a distribution in particle size the probability function [eq. 1.8.2] has to be integrated over the size distribution.

$$R = \int_{-\infty}^{\infty} \psi(x) \int_{-\infty}^{x} \phi(t)dtdx = \int_{-\infty}^{\infty} \psi(x)\Phi(x)dx \qquad \text{(eq.1.8.4)}$$

If pore and particle size distributions are varied independently, there is no significant deviation from the basic curve, i.e. the transition curves of overlapping distributions are virtually indistinguishable from those of a monodispersed species (pores or particles) interacting with a Gaussian distribution of greater width (σ).

Given that in a microemulsion (unlike other liquid/liquid dispersions) the distribution of micelle size is more or less monodispersed (usually even with dissolved enzyme present), the assumption can reasonably be made that the refinement of [eq.1.8.4] is not actually necessary and the pore size distribution has to be contemplated only. Possible deviations can therefore be covered by just increasing the value of σ_p.

2. Materials and Methods

2.1 Materials

Standard chemicals were obtained from the usual providers (Fluka/Sigma-Aldrich, Merck, Roth). Surfactants were provided from the production, namely Dongman Chemicals (Korea) and Condea. Lipases, yADH and HLADH of changing quality were obtained as standard preparations from Fluka/Sigma-Aldrich and Boehringer. FDH and the first batches of CPCR were from non-commercial sources (Forschungszentrum Jülich) that have by now entered the marketplace. Later batches of CPCR came from Jülich Fine Chemicals. The membranes used were obtained from commercial providers but not necessarily from the standard product list. Some were specially modified and provided for the expressed purpose of testing. Ceramic membranes were provided or bought from the Hermsdorfer Institut für technische Keramik (hitk), Kerafol and TAMI Industries, organic polymer membranes from PolyAn. PolyAn also provided samples of a special ceramic-organic composite membrane of Degussa.

The equipment used was for the most part property of the university/institute, some parts for filtration and centrifugation were temporarily borrowed, others produced inhouse.

Enzymes	
Alcohol dehydrogenase from Yeast (yADH)	Boehringer
Alcohol dehydrogenase from Horse Liver (HLADH)	Fluka (0.1-0.32 U/mg)
Carbonyl reductase from *Candida parapsilosis* (CPCR)	Jülich Fine Chemicals
Lipase from *Candida rugosa* (CRL)	Sigma (746-60000 U/mg)
Formate dehydrogenase	FZ Jülich (2.25 U/mg)
Coenzymes	
NADH red. di-sodium salt	Fluka, Boehringer
NAD+	by reduction of NADH, Fluka
NADPH, free acid, di- and tetra-sodium salt	Fluka
NADP+	Fluka
Surfactants	
Tween 85	ICI via Merck
Ethoxylated Tridecanols of the Marlipal 013/x series	Condea (partially donated)
Ethoxylated fatty alcohols of the TDE and LE series	Dongnam Chemicals (Korea) (donated)
Solvents	
Cyclohexane (purum)	Roth, Fluka
Distilled water	desalinated by Millipore plant owned by institute
Glycerol	Fluka
Substrates	
Acetone (tech.grade and puriss.)	Roth, Fluka
Isopropanol (purum)	Fluka
Isopropyl-methyl-ketone (with aromatic contamination)	Merck
2-Butanone	Fluka
2-Octanone	Fluka
2-Octanol (s enantiomer ee >99%)	
Acetophenone	Fluka
1-Phenylethanol (s and racemic)	Fluka
4-nitrophenole	Fluka
4-nitrophenyllaurate (pNPL)	Fluka
Dyes	
Malachite Green	Fluka
Coomassie Brilliant Blue R250	Fluka
PAN (1-(2-Pyridylazo)-2-naphthol)	Aldrich
pH indicators	
Methyl red	unmarked
Bromocresole purple	unmarked
Litmus	Merck
Other chemicals	
Bovine Serum Albumine (BSA)	Sigma
PVA 72000	Merck
Glutaraldehyde solution 25% in water	Fluka
Hydrochloric acid, fuming	Roth, Fluka

Standard Buffer solutions (pH 3, 7, 10)	Roth
Na_2HPO_4	Merck
KH_2PO_4	Merck

Membranes

DisRam Inside® (ZrO_2)	TAMI Industries
Kerafil ($MgAl_2O_4$)	Kerafol
(no specific name) TiO_2, ZrO_2, SiO_2	hitk
PAN (basic and modified by grafting)	PolyAn
Creavis (composite ceramic-organic)	Degussa (via PolyAn)

Equipment

Knick pH meter with glass electrodes from Metrohm and Inlab
Knauer HPLC Pump 46 (346.00)
UVIKON spectrophotometer 943 and 810 (Kontron Instruments)
Laser Diffractometer 4600 SM (Malvern Instruments) with Ar ion laser by Lexel (Mk.95)
IR Spectrum One (Perkin Elmer) with Universal ATR Sampling Accessory
GC (Hewlett-Packard Series 5880A) with chiral column (Chrompack CP-Cyclodex B236M)
Centrifuge Heraeus Varifuge RF
Rotary Evaporator Büchi Rotavapor R-124 with Vacuum Controller B-721
Cross-flow Ultrafiltration unit with magnetic stirrer (produced in institute workshop)

2.2 Methods

2.2.1 Purification of surfactants

The unrefined surfactants were neutralised by the manufacturer before sale. In the past mineral acids were used, today carbonic acids are more common (e.g. citric, malic or succinic acid). This neutralization is only rough and has to be adjusted for our purposes. In order to be used in a microemulsion and with sensitive enzymes involved, the raw surfactants have to be treated and purified.

This involves several steps:

1) removal of impurities and residues from the production process
2) removal of residual alcohol
3) pH adjustment

The unrefined surfactant still contains catalyst (acidic or basic), by-products (esp. if acidic catalyst was used), and varying additives (e.g. buffer salts, odorants) but the main undesired component is residual (unethoxylated) alcohol (up to occasionally 20%).

The surfactants can't be distilled the common way even under vacuum because the boiling points of the residual alcohols (e.g. 150°C/27 mbar for lauryl alcohol or 155°C/20 mbar for tridecanol) are in an area where the ethoxylates begin to decompose (turning brown, later black). A thin layer evaporator with short exposure time can be employed, a rotational evaporator has to work at the limit and produces decomposition products that have to be removed, e.g. with activated charcoal (even very fine membrane filters do not work). Residual alcohol contents can hereby be lowered to less than 1%. Other byproducts and residues have to be removed by other means. In every case the surfactants are dissolved in about a ninefold amount of cyclohexane.

Treatment of the heated mixture with an diluted aqueous solution of acid or base can extract parts of water-soluble impurities and also serve for pH adjustment. The heating increases the hydrophobicity of the surfactant and therefore reduces but does not eliminate losses. This method is very time-consuming due to very slow and incomplete phase separation, though this might be accelerated by centrifugation. Hydrophobic impurities are not affected by this method though.

Those impurities exist either in dissolved or colloidal state and can't be removed by filtration. In older batches of surfactant (before 2000) the colloids are dissolved by short heating and rebuild after about a day. Those have only a minor effect on the formation and water solubilization capacity of microemulsions and can be neglected. In every batch younger than that the effects are massive and have to be dealt with.

Direct separation by freezing out does not work because the mixture solidifies as a whole at about 4-5°C. The most successful method is a combination of cooling, sedimentation, and centrifugation.

The homogenized mixture is cooled to about 6-8°C (fridge) and kept at that temperature for a few hours. Then it is centrifuged at about 8°C for 15-20 min at 800-900 g. The solution is stored in the fridge for another few hours and then again centrifuged. The liquid is decanted and collected, the (semi)solid sediment removed. This procedure is repeated until the solution is completely clear. Fig.X1 shows the result of the progress.

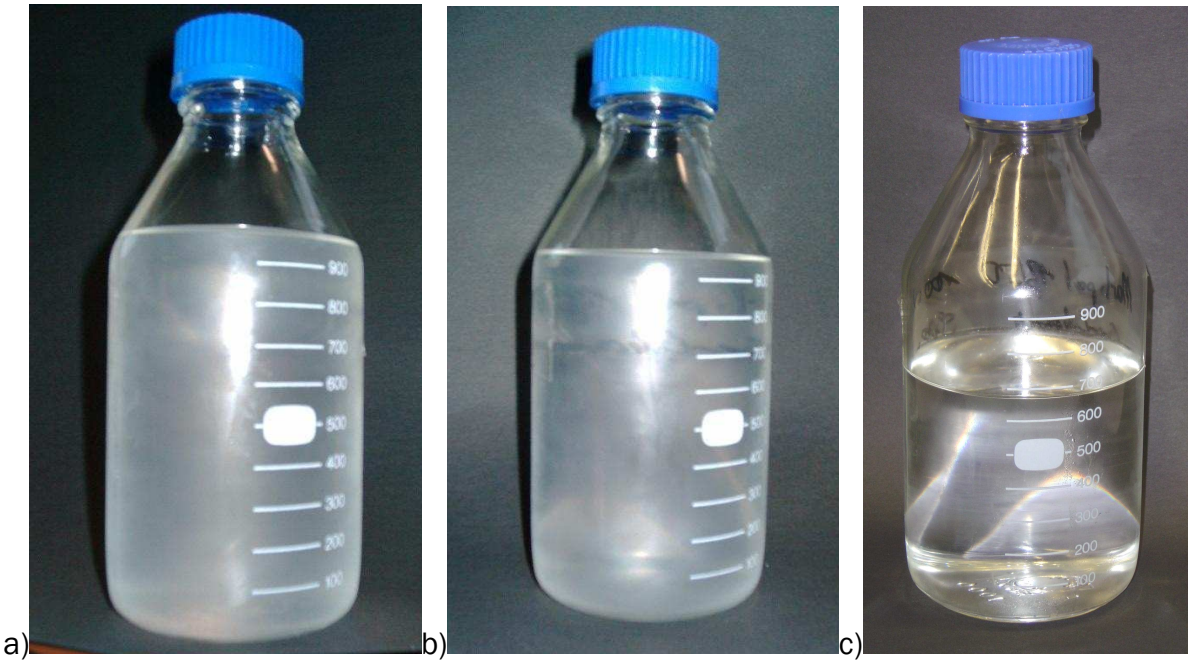


Fig 2.2.1.1: 10 wt-% solution of raw Marlipal 013/50 in cyclohexane
a) before purification
b) after single purification cycle
c) double purification cycle

Prolonged centrifugation instead of rest periods does not have the desired effect. Marlipal surfactants take generally 3-4 turns, those from DONGNAM often require only one [von Dziegielewski 2005].

2.2.2 Determination of the water solubilization capacity of microemulsions

A weighted mixture of surfactant and oil is filled into a cylindrical beaker and stirred. Water is added slowly by burette. The dissolution is observed visually. With the approach of the maximum water solubilization capacity the solution shows a characteristic opalescence.

Exceeding of the limit is indicated by either the occurence of schlieren showing a phase transition towards a liquid crystalline state or a snowlike precipitate of higher density at

the bottom when the stirrer is stopped. From the added amount of water and the given amounts of oil and surfactant the position in the phase diagram can be calculated. By varying the surfactant to oil ratio the 1-phase domain can be determined.

2.2.3 Determination of micelle sizes in the microemulsion

Microemulsions droplets can be considered to be monodisperse. That means that the size distribution of the micelles is very narrow. In the composition range studied here the micelles can also be considered to be of spherical shape. This allows the size determination by straightforward dynamic light scattering (DLS). A sample is illuminated by an Argon laser of 488 nm wavelength and the Rayleigh scattering pattern observed with time with a diffraction photometer (goniometer). The characteristic change of the pattern can be correlated to the Brownian motion of particles of a certain size/mass via the Stokes-Einstein equation under the assumptions made above. From the known characteristics of the micelles (surfactant monolayer etc.) the radius of the water core can be deduced. The instrument used was a Model 4600 SU of Malvern Instruments with an Argon ion laser model 95 of Lexel. The measuring angle was 90°.

2.2.4 Microemulsion formation depending on scale

A mixture of surfactant and oil is filled into the chosen vessel, Erlenmayer flasks or cylindrical reactors (glass beakers) of varying size. A stirrer is installed at the desired height. Depending on size and type of stirrer a number of baffles (between one and four) is installed too. The stirrer is started at the intended speed. The stopwatch is started and the water added quickly. The distinct phases of the microemulsion formation are observed visually and the time for obtaining a homogeneous system taken. The use of an optical dipping probe failed because of the sticky character of the metastable phases formed as intermediates that cover and thus render blind the reflecting surfaces.

2.2.5 Determination of the composition of metastable phases

A mixture of oil and surfactant is prepared and water added under only slight stirring. Thus metastable phases are formed that under more vigorous stirring might transform into microemulsions. Once the macrodispersion is complete the stirring is stopped and a portion of the sedimented metastable phase extracted. Short centrifugation may be applied to remove residual liquid but this also risks a change in composition.

The sample is weighted and then distilled slowly under atmospheric pressure in a closed system. The surfactant remains in the distillation bulb while oil and water are collected in

the (cooled) receiver where a phase separation takes place. By backweighing the composition of the initial sample can be determined. The best results are obtained by dividing the sample and destilling it once with excess water and once with excess oil. This allows an estimation of the usually inevitable loss of volatile compounds (oil at a higher rate than water). If the sample is allowed to "age" for some time before the distillation, the macroscopic characteristics can change significantly due to oil evaporation.

2.2.6 Supply and handling of available enzymes

Enzymes are commercially available in different forms with or without additives. The most common form of supply are lyophilized powders and glycerol/water solutions. The properties of those can and do differ for every batch even when obtained from the same provider. The four enzymes mainly used in this work each represent a different form.

1. alcohol dehydrogenase from horse liver (HLADH) [Larsson 1987]
2. carbonyl reductase from *Candida parapsilosis* (CPCR) [Peters 1993 (1+2)]
3. formate dehydrogenase from *Candida boidinii* (FDH) [Neuhauser 1998]
4. Lipase from *Candida rugosa* (CRL) [Benjamin 1989, 2001]

CRL was available as a completely soluble highly active (>>100 U/mg) lyophilized powder. Different batches could differ in activity to a degree but provider declarations were higly reproducible. Storage durability was high.

HLADH was available as a lyophilized powder with varying degrees of solubility and generally very low activity (0.1 U/mg). A separation of insoluble parts (s.b.) was frequently necessary sometimes combined with loss of activity. Solutions had to be tested under standard conditions routinely for reproducibility to be ensured. Contamination with other enzymes could not be excluded. Nondeclared additives (e.g. buffer salts) could have influenced experiments, especially concerning in the microemulsion environment. Storage durability in powder form was medium, in solution low.

CPCR was available as a clear glycerol solution of varying activity from medium to high (5 to >100 U/ml) but with good reproducibility. Older batches from natural sources still contained contaminations with other enzymes (e.g. malate dehydrogenase) that resulted in a small extra loss of coenzyme even in absence of standard substrate. New batches from genetically modified microorganisms did not show that effect. Storage durability was medium to high.

FDH was available as a crude brown dispersion in glycerol (ca. 80 U/ml). Because only very small amounts were usually needed, a purification was deemed unnecessary.

Reproducibility and storage durability were excellent (no detectable activity loss after 5 years).

For purification an aqueous solution/dispersion was centrifuged for a few minutes and the precipitates discarded. The liquid was then tested for activity. A varying loss of activity was usually detected. Both lyophilized powders and glycerol solution have certain advantages and disadvantages. The absence of solvents in the first case avoids possible undesirable interactions with the solvents in the actual reaction system. In the reverse micellar system even a relatively small extra amount of water can mean a critical waste of capacity. Glycerol can act as cosurfactant and thus influence the basic structure of the system in a negative way. Solutions also mean a fixed maximum concentration while powders allow an easier variation and are limited mainly by maximum solubility (the solutions are rarely saturated). On the other hand the powders have the disadvantage of partial reversible denaturation of the enzyme and often require a period of renaturation, i.e. the enzyme may not be usable instantly (cf. [Rees 2000]). The main factor here is the need for a regeneration of the hydration of the protein that was lost during the freeze-drying process and the need to overcome the attraction between the protein molecules that often form semi-stable clusters when bereft of solvent. Those clusters show a significantly lower activity than the isloated enzymes, an effect that (although to a lower degree) can be observed in concentrated solutions too.

While the declared activity of solutions is usually reliable, the activity of lyophilized powders has to be carefully checked after solubilisation, especially when insoluble residue remains. The full solubilisation may take significantly longer than the apparent dissolving time. Produced stock solutions should therefore be repeatedly tested for activity changes.

2.2.7 Determination of protein content

For the liquid dispersions of enzyme (solvent: glycerol/water) the total protein content has to be determined in order to relate activity to amount of catalyst. This was done by the Bradford method based on the formation of a complex of a dye with protein. A sample of unknown protein content is compared with another of known content [Bradford 1976].

Reagents:

10 mg of Coomassie Brilliant Blue R-250 in 5 ml ethanol

10 ml 85 wt.-% orthophosphoric acid

filled up to 10 ml with deionized water

Protein standard:

100 mg BSA (bovine serum albumine) + 900 mg NaCl in 100 ml deionized water

For calibration 5 ml of reagent solution are mixed with 20-100 µl of protein standard filled up to 100 µl with pH7 phosphate buffer.

After exactly 5 min the mixture is spectrophotometrically measured at 595 nm in quartz cuvettes with the reference cell holding the same amount of reagent with 100 µl buffer. Sample and reference have to be prepared simultaneously from fresh reagent mixture because the absorption changes with time. The calibration thus produced gave 1.12 +/- 0.03 mAbs per µg BSA. The value given in [Bradford 1976] differs by almost exactly the factor 10 hinting at a misprint.

If samples with unknown protein content are outside the linear segment of the calibration curve and/or show an absorption beyond the linearity of the spectrometer, they have to be diluted appropriately.

Enzyme solution is appropriately diluted and treated as described above with 10 µl enzyme dilution + 90 µl buffer. As described in [Bradford 1976] the protein-dye complex tinges the quartz surface of the cuvettes. It can't be removed with water but washes off, if acetone is used.

2.2.8 Determination of Enzyme Activity

In order to determine the initial activity of the enzyme dependent on the microemulsion composition, oil and surfactant are premixed in a quartz cuvette. Then the water soluble components (buffer etc.) are added and the cuvette shaken in order to form a microemulsion. The sequence of adding substrate, enzyme and coenzyme (in case of ADH or FDH) may depend on the distance of the final microemulsion from the phase boundary of the one-phase region. The enzyme is typically added last because the dissolving of a component in an already formed microemulsion is fast (thus avoiding partial deactivation during mixing). Near to the boundary of the stable microemulsion region the mixing time increases again, so the enzyme is better added as the penultimate compound. Solid compounds are predissolved in either oil or water before adding them to the mixture. Using a spectrophotometer (UVIKON 943) the change of extinction at certain characteristic wavelengths is measured with time. In case of ADH/FDH this is usually the range between 340 and 370 nm where the coenzyme NADH absorbs but NAD+ doesn't. An alternative is the absorption of the substrate ketone in case of ADH. In case of lipases (no coenzyme) the absorption of p-nitrophenol (at 410 nm) formed as product of the hydrolysis of pNPL is used. Especially in the case of aromatic substrates the ketone

bands can overlap with the NADH bands. In that case it can be necessary to remove samples, stop the reaction (e.g. by instant freezing or chemical or thermal enzyme deactivation) and to determine the conversion by gas chromatography. The rate of change in extinction with time can be correlated to the reaction rate either by using known extinction coefficients or comparison to calibrated samples. The initial slope, taken after a short stabilization period (< 30 s), is usually sufficiently linear to yield the reaction rate.

2.2.9 Determination of enzyme deactivation during formation of microemulsions

Water and oil are mixed together with a certain amount of enzyme in a centrifuge tube.The mixture is then either shaken by hand or put on a vibrator table for a measured time. The mixture is then quickly separated by centrifugation and a sample of the aqueous phase spectrophotometrically tested for enzyme activity. The measured rate is compared to the rate of fresh enzyme in water. The deactivation time, the water to oil ratio and the order of mixing are varied. The enzyme may be predissolved in the aqueous phase, dispersed in the oil phase before the addition of the water or injected through the oil phase into the water. The separation may be accelerated (or facilitated in the first place) by adding either extra water or oil before the centrifugation. Highly viscous mixtures of water/oil/protein often do not separate completely during centrifugation. It is important that no metastable oil/water/protein phase remains when drawing the sample for activity measurements because enzyme is absorbed in this, thus reducing the amount of enzyme in the water phase resulting in an apparent higher loss of activity.

2.2.10 Determination of long-term deactivation of enzyme in microemulsion systems

A stock solution of a microemulsion is prepared with ADH dissolved together with a small amount of substrate (acetone) and coenzyme (NADH). The solution is thermostated and protected from daylight. In increasing time intervals samples are removed and filled up with a microemulsion of identical γ and w_0 value containing substrate and coenzyme in concentrations above enzyme saturation but below strong enzyme inhibition. The activity is determined spectrophotometrically and compared to the initial activity.

In order to exclude a non-catalytic reaction (e.g. natural coenzyme decay) from falsely indicating residual activity, the remaining stock solution is thoroughly heated at the end of the experiment and tested again (60-70 °C for at least 20 minutes).

2.2.11 Determination of coenzyme stability

NAD(P)H is dissolved in water, buffer solution or microemulsion. The solution is thermostated and kept from light. The change of the absorbance at 340 nm is measured with time. In order to distinguish between reversible oxidation (NAD(P)H → NAD(P)+) and irreversible decay, a small amount of FDH and formate can be added at the end and the restored absorption be compared with the initial.

2.2.12 Preparation of PVA solutions

It is useful to produce a stock solution of higher concentration (10-20 wt-%) that is diluted for the actual experiments. The powdered PVA is weighed into a round bottom flask of at least twice the volume of the intended solution and filled up with the appropriate amount of deionized water. The mixture is boiled under reflux (100 - 130°C) and slight stirring for at least 40 min and then allowed to cool to room temperature. In case of strong foaming (esp. at high PVA concentrations) stirring and heating has to be adjusted. The slightly yellow solution can be stored for several weeks but should be reboiled occasionally and before experiments in order to suppress the growth of microorganisms (mould).

2.2.13 Preparation of PVA gels

PVA gels can be produced by cycles of freezing and thawing with or without a crosslinking agent or at room temperature (only with crosslinking). Non-crosslinked gels are prone to aging and will dissolve with time, if brought in contact with water. Crosslinked gels are more stable.

1. Gel without crosslinking by freezing-thawing method

The PVA solution is poured into a mould, e.g. a crystallizing dish, or spread evenly on a support, e.g. filter paper. The cast is put into a freezer (-6 to -18°C) for 30 minutes and then allowed to thaw for another 30 minutes. This freezing and thawing cycle is repeated several (at least 5) times.

2. Gel with crosslinking by freezing thawing method

PVA solution is mixed with GDA solution (25% in water) under stirring or shaking, poured onto filter paper support moisted with 0.1-1 wt-% HCl and evenly distributed to form a thin membrane. The weight difference is noted, the supported membrane put in the freezer and treated as in the case of the un-crosslinked gel.

3. Gel with crosslinking at room temperature

A PVA solution is mixed with GDA solution (25 wt-% in water). Under vigorous stirring a volume of concentrated/fuming HCl is added equal to 50-100% of the GDA volume used.

The mixture is poured into a mould or evenly distributed on filter paper support. This has to happen fast because the viscosity of the mixture increases rapidly once the acid is added leading to solidification. The cast is covered but with enough space above to allow the evaporation of excess water. Depending on the amount of GDA and acid used, the initial solidification (no flow/change of form when upturned) can be complete in 20 minutes or take more than a day. In a second step the gel loses excess water and shrinks. In order to avoid this, the gel should be kept moist. The process is partially reversible at medium GDA:PVA ratios (about 1 molecule GDA per 20-100 PVA monomers), at high GDA:PVA ratios (>1 :10-20) the drying is irreversible. Fig.2.2.13.1 shows the casting form and distributor roll together with a successful and a failed sample (due to excess crosslinker and too thick a gel layer).

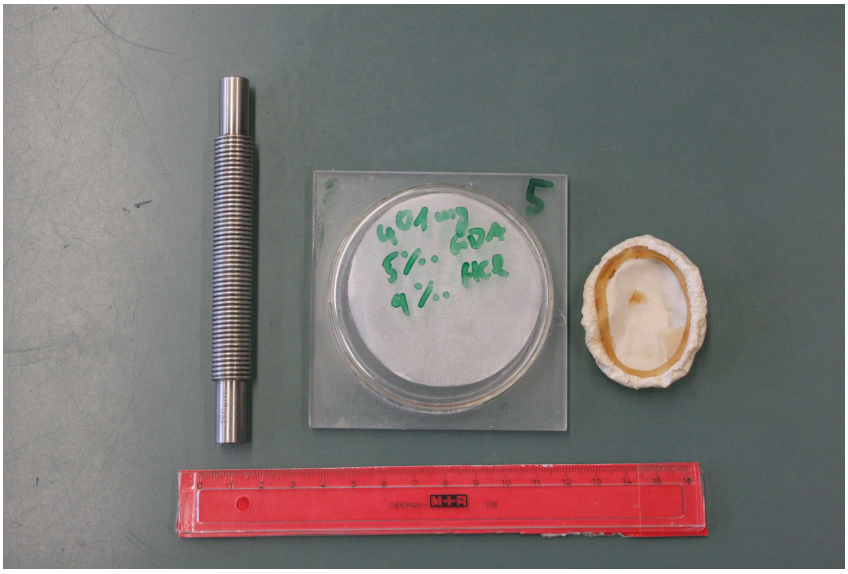


Fig 2.2.13.1: Samples of crosslinked PVA gels (RT method) on filter paper support
left: steel roll used for distribution of gel on support
center: mould with paper supported PVA membrane
right: failed attempt as result of overdosed crosslinker and overthick layer

2.2.14 Lipase immobilization on supported PVA membranes or pure PVA foils

The dried membrane or cut PVA foil is immersed in a 50 mM phosphate buffer solution (pH 8) of *Candida rugosa* lipase (c_E =2-20 mg/l at 60kU/mg) for 30 minutes at room temperature by use of a seesaw table. Then a small amount (100 µl) of 25 wt-% in water GDA solution is added and allowed to react for a further 30 minutes. The membrane/foil is then removed from the bath and rinsed thoroughly twice with distilled water (10 ml each). In order to bind remaining GDA the rinsed membrane/foil is then immersed for another 30 minutes in a 1 wt-% solution of BSA. The membrane is now put into the ultrafiltration cell and rinsed with 0.5 l deionized water in order to remove unbound enzyme and adsorbed BSA. In case of pure PVA foil, i.e. without support, the strips are put

between two pieces of filter paper in membrane size before insertion into the ultrafiltration cell. The rinsing solutions can be checked either for enzyme activity (hydrolysis of p-NPL) or directly spectroscopically at 280 nm in order to determine the ratio of immobilized enzyme to unbound enzyme. The enzyme loading is then defined by the mass of immobilized enzyme (mg) divided by the volume of PVA solution used for the cast (l). Loss of water during the gelation is ignored. The method is unsuitable for ADH.

2.2.15 Assay for immobilized lipase

A stock solution of 85% water, 10% buffer solution (50 mM phosphate, pH 8) and 5% nonionic surfactant (either of the Marlipal series or the equivalent from DONGNAM Chemicals) is prepared (solution A). Varying amounts of the substrate p-NPL (20-200 mg) are dissolved in 1 ml cyclohexane each (solution B). 200 ml of substrate solution of desired concentration are produced by mixing appropriate volumes of solution A and B (solution C). The solution C is fed through the membrane or foil grid in an ultrafiltration cell in dead-end mode at varying flow rates. The conversion of p-NPL into p-nitrophenol and lauric acid is determined by measuring spectroscopically the absorption of p-nitrophenol at 410 nm (the absorption is dependent on the pH value that therefore has to be tightly controlled). This can either happen by collecting samples in short time intervals or by pumping the permeate through a flow-through cuvette. In the first case the sampling time has to be constant and short because leaching enzyme can falsify the measured conversion suggesting too high a value because the reaction proceeds in the bulk solution. (Methods in chapters 2.2.12-15 modelled after [Djennad 2003] except room temperature method of gelation)

2.2.16 Spectra of pH indicators

Stock buffer solutions covering the transition interval of a pH indicator are prepared. Small amounts of indicator are mixed with the buffer solutions and the spectra are measured with a photometer. The absorption maxima are determined and the isosbestic point used to normalize the spectra allowing them to be used for calibration. This sequence is repeated with the buffer solutions as aqueous part of microemulsions. In order to avoid data distortion through alkaline or acidic impurities in the surfactant used it is necessary to test the surfactant beforehand (e.g with the Malachite Green test [El Seoud 1980] for traces of alkaline contamination). Raw surfactant may also have buffer capacity strong enough to "overrule" the limited amount of stock buffer in the microemulsion. This may require the purification of the surfactant by the method described above.

2.2.17 Test for bleaching sensitivity of pH indicators

A saturated or at least highly concentrated aqueous solution of the pH indicator is prepared. From this pairs of sample solutions are produced that have an absorption within the linearity interval (i.e. the Lambert-Beer law applies). Those samples are pure aqueous/buffer solutions, the same with a certain amount of dissolved surfactant and w/o microemulsions. Of every pair one sample is kept in the dark, the other exposed to strong daylight. From time to time the residual absorption of the sample solutions is measured spectrophotometrically. It is also tested, whether the indicator is still active. For this a small amount of the sample solutions is treated with strong acid/base and checked for the characteristic colour change.

2.2.18 Measurement of membrane retention

The membrane to be studied is placed in a cross-flow ultrafiltration cell and a microemulsion pumped in a loop (Fig.2.2.18.1). The permeate is analyzed by different methods.

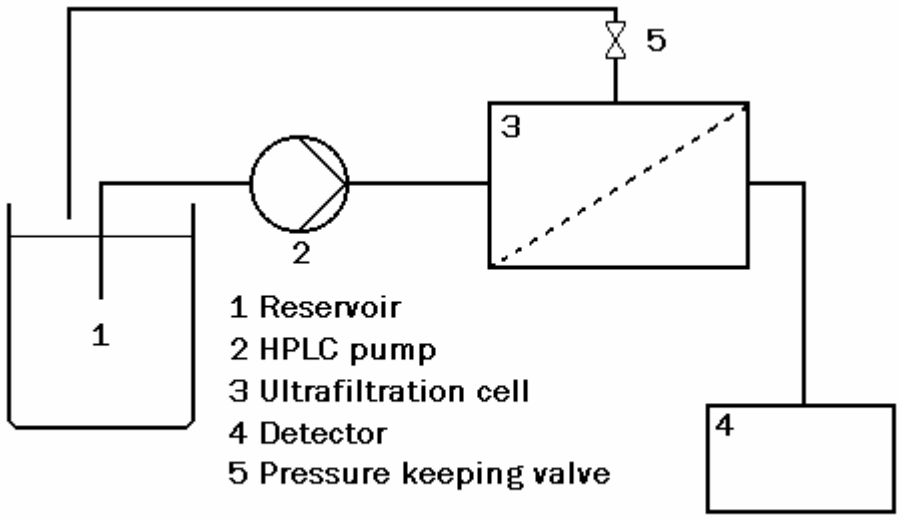


Fig 2.2.18.1. Flow scheme of ultrafiltration apparatus. The UF cell can contain either a filtration or reactive membrane. If the valve (5) is closed completely, the system is in dead-end mode, otherwise in cross-flow mode

The pressure in the cell is held constant by a valve of variable resistance. The main point of interest here was not the relation of pressure to permeate flow but the retention of the dispersed phase and/or compounds dissolved therein. Therefore the permeate was tested for the concentration of water and oil-insoluble model compounds (see below). In reverse experiments an o/w microemulsion with a water-insoluble dye was used. The

actual insolubility of the model compounds/dyes in the opposite phase was tested using extensive stirring and ultrasonication.

As water-soluble compounds Coomassie Brilliant Blue R250 and Malachite Green were used, as oil-soluble compound 1-(2-pyridylazo)-2-naphthol (PAN). The dyes were analyzed by spectrophotometry using an UVIKON 943 of Kontron Instruments. In case of Malachite Green the pH value has to be controlled and kept slightly acidic because the dye bleaches under basic conditions (test substance for alkaline impurities).

The water content of the samples was analyzed either by IR spectroskopy or by Carl-Fischer titration. For the former method the IR spectrum between 3700 and 3050 cm^{-1} is integrated and compared to samples of known composition. At the low concentrations encountered here this is rather imprecise but has the advantage of being quick and not in need of hazardous chemicals. The latter method is very precise with errors < 1% but time-consuming and wasteful. It was therefore only used in the rare cases where this precision was crucial. After the retention tests the dye containing microemulsion is replaced by either a dye-free one or pure water or oil and the permeate checked for residual dye that had been reversibly absorbed at the membrane.

3. Results and Discussion

3.1 Determination of the phase boundaries of the one-phase microemulsion

Previous studies (Berger 1999; Orlich 2000) have shown that ADH activity and stability in microemulsions are best at high w_0 values, i.e. large micelles. This has the disadvantage of being near the phase boundary of the single-phase region. With the surfactants applied here an exceeding of the water solubilization capacity does not necessarily result in the formation of a second phase but an internal transformation towards e.g. a lamellar structure that is detrimental to both activity and stability of the ADH enzymes, as well as for mass transfer of substrate. Therefore it was necessary to determine the phase boundaries within the phase diagram.

Due to limited supply of purified surfactant and the aim for reuse, only the phase boundary for the system surfactant/cyclohexane/pure water will be experimentally discussed here. As older studies (e.g. Berger, Orlich) have shown, the water solubilization capacity shrinks significantly with the addition of dissolved salts or protein. At higher salinity the phase transition tends to be shifted towards a two-phased system instead of or in addition to the lamellar structure.

In order to determine the maximum water content depending on the composition, different mixtures of oil and surfactant (TDE-7 of Dongnam Chemicals) were prepared and slowly titrated with deionized water. The mixture was stirred vigorously during that process. Visible signs for the approach of the boundary are increased time needed to solubilize the water (instantly at the beginning, up to ten seconds at the end), increased viscosity, and opalescence. The transition is marked by slight turbidity and/or the occurence of visible schlieren. The end point is rather sharp at low and high surfactant concentrations but very fuzzy at γ values of 6-7%.

In Fig. 3.1.1 the maximum w_0 values are plotted against the γ value, in Fig. 3.1.2 the ternary phase diagram shows the boundary of the 1-phase region.

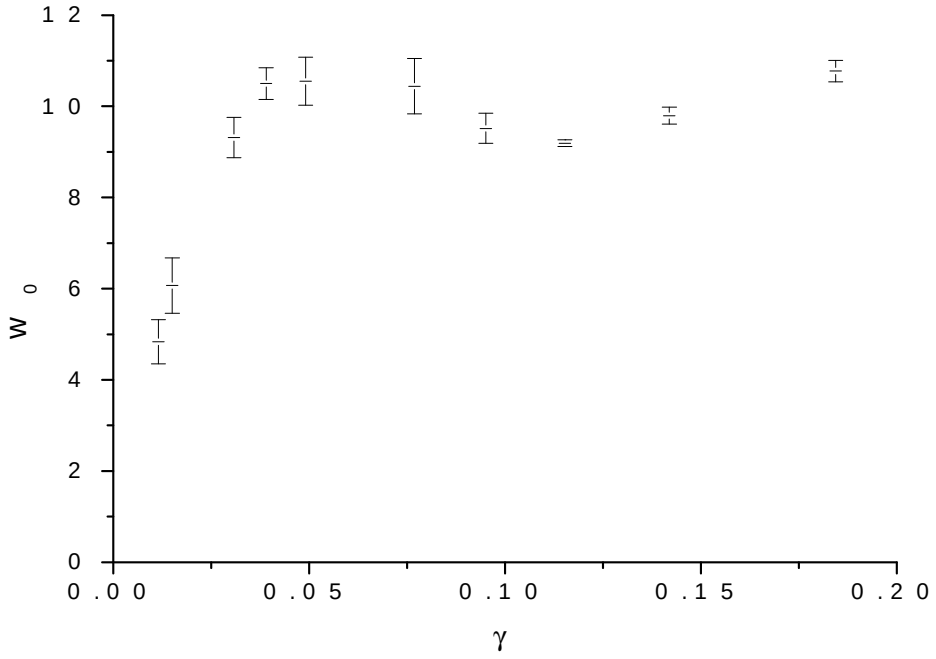


Fig. 3.1.1: Water solubilization capacity of a microemulsion from TDE-7/cyclohexane/water

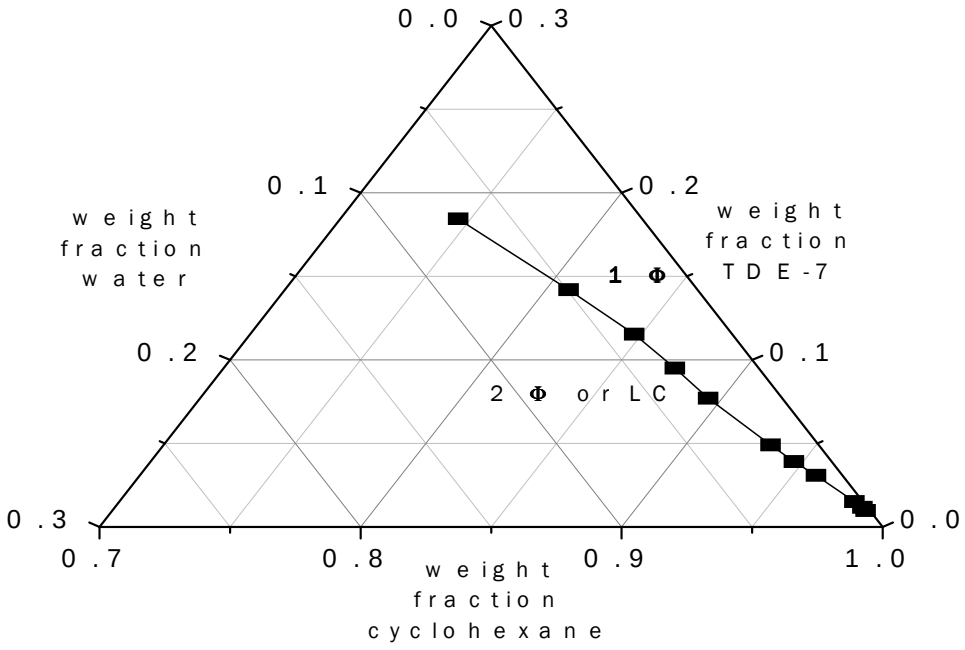


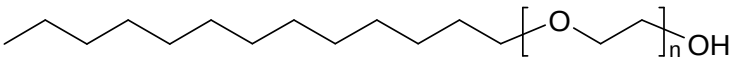
Fig 2.1.2: Oil rich corner of phase diagram of a microemulsion from TDE-7/cyclohexane/water

As can be seen in Fig.2.1.2, the phase boundary curve crosses zero at a γ value > 0. This marks the cmc, in effect the amount of surfactant dissolved molecularly. Only above this

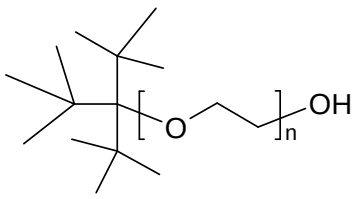
point micelles are formed and water can be dissolved (miscability of oil/water is far lower and can be neglected). The cmc can hereby be estimated to be about 0.5 weight percent for this surfactant in cyclohexane.

Interestingly the $w_{0,max}$ value goes through a maximum at about γ 6-7% (also point of least sharp transition) and a (local) minimum at about 12% (also point of sharpest transition). Comparision to experiments carried out with the similar Marlipal 013/60 (Berger 1999) suggest a correlation to the activity jump of ADH enzymes that would correspond with the former point. The reason for this has not yet been determined.

The maximum solubilization capacity for pure water for this surfactant is about 10 times the molecular amount, i.e. 10 molecules of water per molecule of surfactant. This is significantly below the values achieved with comparable Marlipal surfactant of older formulation and can be contributed to both the degree of purification (esp. removal of residual alcohol) and branching (natural unbranched for the Dongnam products, highly branched for "old" Marlipal, cf. Fig. 3.1.3).



ethoxylated alcohol from "natural" sources



ethoxylated alcohol from synthesis
with maximum branching

Fig. 3.1.3: Nonionic surfactants based on different isomers of tridecanol showing minimum and maximum branching resp.

3.2 Micelle Size

Microemulsion droplets can normally be considered to be monodisperse, i.e. the size distribution of micelles is very narrow and primarily dependent on the microemulsion composition parameters (γ, w_0, cμc) and the choice of components [(co)surfactant(s), oil, water, dissolved compounds].

Because the normal micelle size is in the nanometer range and the exchange rate in the millisecond range, information about the actual size can only be obtained by indirect methods, not by direct observation. Theoretical approaches include the deduction of

micelle concentrations (number of micelles per volume) from macroscopic characteristics (e.g. viscosity) and micelle geometry from molecular data (surface energy or spatial demands). The most common experimental approach is the specific scattering of monochromatic light (dynamic light scattering DLS), x-rays (small angle x-ray scattering SAXS) or neutrons (small angle neutron scattering SANS). Through the Stokes-Einstein equation the hydrodynamic diameter can be calculated.

$$d = \frac{k_B T}{3\pi\eta D} \qquad \text{(eq.3.2.1)}$$

Under the assumption of a certain micelle geometry (here: spherical) and the presence of a surfactant monolayer, the relative size of water core and surfactant shell can be calculated. Micelle concentration data for given γ and $w_{0,1}$ [Berger 1999] yield an average thickness of the surfactant monolayer (r_t) of about 1 nm. Orlich [2000 (1)] reports values between 1 and 2 nm.

This model does not take into account that (for the surfactants used) a water core is not distinguishable up to a w_1 value of about 4 because the water molecules are used up for the solubilisation of the polar head groups. A similar partial penetration of the outer surfactant shell by solvent molecules is also to be expected. Thus the outer and inner border of the micelle are in reality by far more fuzzy than the simple core-shell model implies. The irregular surface of the micelle has been aptly likened to a sweetgum seed [Michaels 2006]. The hydrodynamic diameter of micelles as obtained from scattering data could therefore be expected to slightly exceed that calculated from simple volumetric/geometric data considerations.

3.3 Measurement of the pH value in the microemulsion system

The seemingly easiest way to observe the progress of the reaction is the determination of change in the pH value of the reaction system. The reduction of one ketone molecule correlates with the formal production of one unit of alkaline hydroxide or the elimination of one unit of formic acid in case of the FDH regeneration route. This means that the pH value of the aqueous part of the microemulsion increases with the progress of the reaction. Because of the strong dependence of enzyme activity and stability of both enzymes and co-enzymes on the pH value, it has to be monitored carefully. The reaction system should under normal circumstances be kept within ± 0.2 from neutral (ph 7) and must be kept within about ± 0.5 for enzyme activity and coenzyme stability. In order to keep the parameters of the microemulsion constant (as is necessary for a continuous process), the pH value can only be adjusted (downwards) by addition of pure formic acid

that, by conversion to carbon dioxide, does not cause a net change in the microemulsion composition. The coenzyme level is to be kept constant in the continuous process and therefore gives no clue as to the progress of the reaction. Because the FDH still works at pH values higher than those acceptable for the available ADH, there is no "warning light" drop in co-enzyme levels signalling the necessity to add fresh formate (the same holds true for formate overdosing).

The use of buffers with high capacity in order to keep the pH value from regions deleterious to the enzymes is problematic. It would decrease the water solubilization capacity ($w_{0,max}$) of the microemulsion in the desired one phase region. Our enzymes however show their highest activity and stability at high w_0 values. In addition, the coenzymes show strong sensitivity to several common buffers, especially phosphate based ones [Rehm 1998]. The exclusion of strong buffering in combination with the necessity to use strong acid only for pH change compensation makes the system too sensitive for working without permanent monitoring. The peculiar characteristics of microemulsion systems impose considerable restrictions on standard pH measurement procedures. The presence of surfactants, although problematic, does not make pH sensitive electrodes unusable per se. Specialized equipment has been developed by industry for reliable use in surfactant-rich environments. But those are intended for use in systems with an aqueous or at least protic or (at minimim) higly polar continuous phase (e.g. acetic acid, ethanol). The use of an apolar continuous phase (w/o-microemulsion) and nonionic surfactants does not meet these criteria. Consultations with Metrohm corroborated the conclusion that the use of pH sensitive electrodes is not technically feasible for the intended reaction media. That leaves only the possibility of using spectrometric methods for the continuous or at least semi-continuous determination of the pH value. That means the use of chemical pH indicators. This creates a whole new set of problems along the lines of

1. addition of another component to an already "crowded" sensitive system
2. distribution of the pH indicator within the microemulsion
3. influences of the pH indicator on other components and the phase behaviour
4. influences of other components on the pH indicator

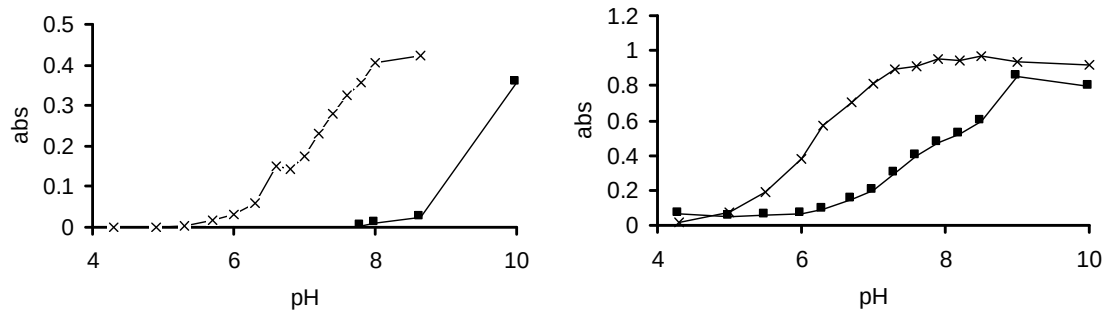
This list does not include the question of variable caustic or acidic contaminations of the surfactant, a topic that has been dealt with in chapter 2.2.1 .

As has been shown earlier, activity and stability of the ADH enzymes are highest at or near the maximum w_0 value of a given microemulsion system. Each extra component dissolved in the aqueous micro-phase (the reverse micelles) of the microemulsion results

in a reduction of $w_{0,max}$. Compared to a conventional aqueous bulk solution, water constitutes only a small relative amount of a w/o-microemulsion (usually < 10%, often far less). The absolute amount of indicator dye necessary to achieve the same extinction on the other hand is unchanged, requiring a far higher relative concentration in the aqueous micro-phase. In the case of litmus this actually excludes its use, because the necessary concentration exceeds the acceptable due to its low coefficient of extinction. In order to keep the concentration low enough while still obtaining a strong enough response, the light-path in the measuring cell would have to be extended by an order of magnitude.

Even disregarding the effect of highly concentrated indicator (salts) on the phase behaviour, an adverse effect on the enzymes (or vice versa) can't be excluded from the outset. In our experiments we could not detect any indicator loss through enzymatic conversion (the bleaching discussed below is caused by other effects). A possible inhibition or accelerated deactivation has not been sufficiently tested yet to give a definitive answer.

The micro-environment in the reverse micelles shows strong differences compared to bulk aqueous solution in several aspects. This also influences the behaviour of pH indicators, and, unfortunately, in a quite unpredictable manner. As El-Seoud & al. have shown (El Seoud 1983, 1989; Vieira 1991; Chinelatto 1991), the relevant parameters of pH indicators (coefficient of extinction, position of absorption maxima and isosbestic point, pKa value) can vary significantly depending on the used surfactant and the microemulsion parameters. The latter can be explained easily by the different physical water structure in the micelles and is predictable to a certain extent.



a) b)

Abb. 3.3.1 a) Absorption of bromothymol blue (6,7 mg/l) in water (X Abs_{Max} at 616 nm) and in a microemulsion from 013-60 (detopped)/ cyclohexane / water with γ = 0,1 and w_0 = 5 (■ Abs_{Max} at 626 nm). b) Absorption of bromocresol purple (3,3 mg/l) in water (X Abs_{Max} at 589 nm) and in a microemulsion from 013-60 (detopped)/ cyclohexane / water with γ = 0,1 and w_0 = 5 (■ Abs_{Max} at 599 nm). [Orlich 2000]

The effect of the chosen surfactant on the other hand has not been described yet (to our knowledge) theoretically but seems still to be merely empirical, i.e.: for each pair of surfactant and indicator, the parameter shift has to be determined experimentally. An example can be seen in Fig. 3.3.1. The distribution of the indicator in the system is therefore likely an important factor with a low effect on compounds concentrating at the core of micelles ("pool of normal water") and a high effect on those with an affinity to the surfactant. This distribution also plays a role in the question of the filtration of the reaction system for product extraction.

Unfortunately, the choice of indicator depends on another factor not yet discussed. The tested pH indicators that most closely meet the desired interval of color change, have shown to be sensitive to bleaching, i.e. the loss of the indicator property. In the case of bromocresol purple the product of this decay is not colorless but prtends a nonexistant pH value of about 7.5, i.e. outside the desired area (=> misleading).

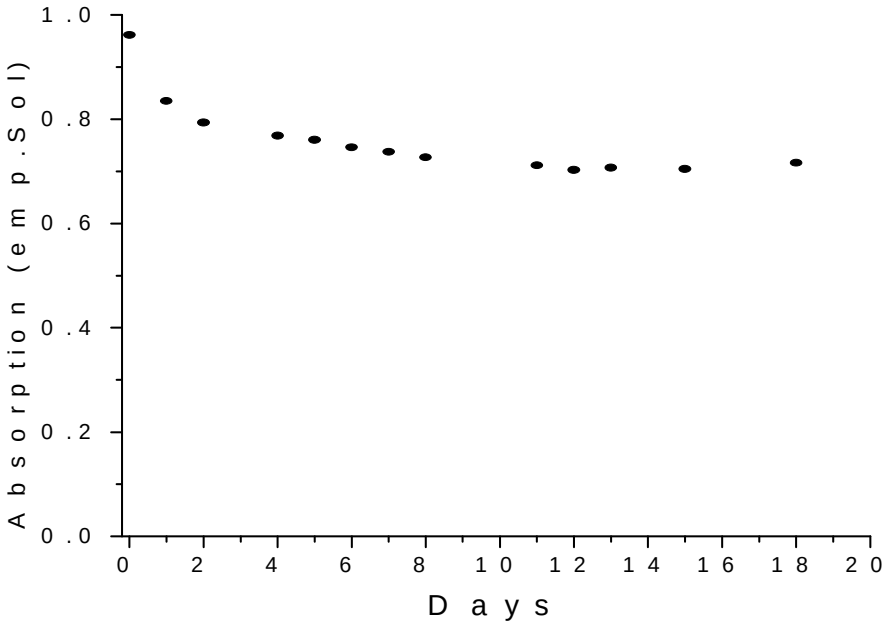


Fig.3.3.2: Bleaching of litmus solution with time. Solution exposed to daylight, not directly to sun

If the indicator has to be a permanent part of the reaction medium, a compound not sensitive to bleaching has to be found, the alternative being a discontinuous pH testing by sampling (e.g. flow injection method) with an unavoidable loss of substance. Litmus has shown a partial bleaching of about 20% (Fig.3.3.2) over a period of about four days. After that it seems to be stable, provided it is not exposed to strong light for extended

periods of time (normal daylight seems to have little effect). For reasons quoted above, litmus had unfortunately to be excluded from further consideration.

Experiments by Orlich [2000(1)] and those in the early stages of the current work were carried out with "detopped" Marlipal 013/60 (cf. chapter 2.2.1), those by von Dziegelewski [2005] mainly with partially refined Marlipal 013/50 as surfactant. Apart from a different pH adjustment by the manufacturer (cf. chapter 2.2.1) there is a measurable, albeit small, shift in the spectrum of bromocresol purple. In both cases the pKa shift in comparision with normal aqueous solutions is towards the basic end, i.e. the color change takes place at higher pH values. Other indicators (methyl red/orange, phenolsulfonphthalein, bromothymol blue) were tested but showed color change outside the desired range. Bromocresol purple solutions without surfactant are moderately stable towards daylight. If surfactant (ethoxylated fatty alcohols, technical grade) is added, the solution begins to bleach slowly. This effect is accelerated but not caused by daylight, solutions kept in the dark also decay but more slowly. In any case, the indicator characteristics are lost within less than a week, normally in about 3 days. The surfactant was tested for the presence of peroxides as a possible reason but none could be detected (positive tests only with deliberately contaminated samples). Bromocresol purple is therefore unsuitable as permanent additive to the microemulsion system. It can be used for testing of samples taken from the reaction system, e.g. in a flow-injection analysis.

In a communication Prof. El Seoud (Univ. São Paulo, Brazil) recommended alkaline salts of dinitrophenolsulfonic acid as pH indicators not sensitive to bleaching. These substances are also completely insoluble in apolar media, preventing a loss via filtration of the oil phase (as would be the case with most other investigated compounds).

A selection of possible substances is shown in Fig. 3.3.3. In theory the pKa can be fine-tuned by the relative position of the substituents.

The practical problems lie in the selective synthesis of these compounds [Post 1874]. While the mononitro-derivatives (wrong pKa) are either commercially available directly or easily obtainable from compounds that are, the dinitro-derivatives are not. Synthetic procedures described in literature [Post 1874; Ullmann 1909; King 1921; Marqueyrol 1920, 1921] give very low yields (7-11%) and usually produce a mixture of regioisomers requiring a separation (with picric acid as a major byproduct). Furthermore, the introduction of the second nitro-group turns the compounds from mere irritants to sensitive explosives that can't be handled dry safely. For these reasons we refrained from following that route.

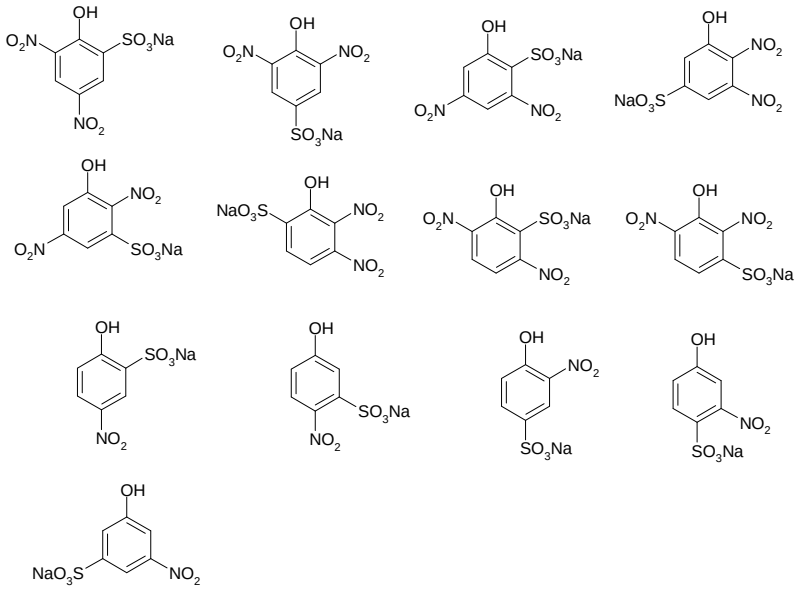


Fig. 3.3.3: Selection of possible pH indicators of low sensitivity to bleaching

Conclusion: The continuous determination of the pH value in a w/o-microemulsion remains a problem not yet finally solved. As preliminary solution an external testing of samples with bromocresol purple as indicator is proposed.

3.4 Activity of ADH in microemulsions

3.4.1 Enzyme in ME compared to water

In the microemulsion the local enzyme concentration within the droplets at equal overall concentrations is much higher than in bulk water because the aqueous phase of the microemulsion takes up only about $^1/_{40}$ of the total volume. At the same time the amount of the (expensive) co-enzyme necessary to saturate the enzyme is much lower because for the local concentration the same 1:40 relation applies. So, on first view the volume related enzyme activity can be achieved at far lower expense. But the higher local enzyme concentration has also an undesired effect. The total activity is not strictly proportional to the amount of enzyme per volume but the activity per enzyme unit decreases. In water the effect can often be neglected because the critical concentrations are higher than can be economically afforded. With HLADH in water it was not observed at all, or it was at least smaller than the natural variation. CPCR loses about 20% of relative activity compared to a highly diluted solution (Fig.3.4.1.1).

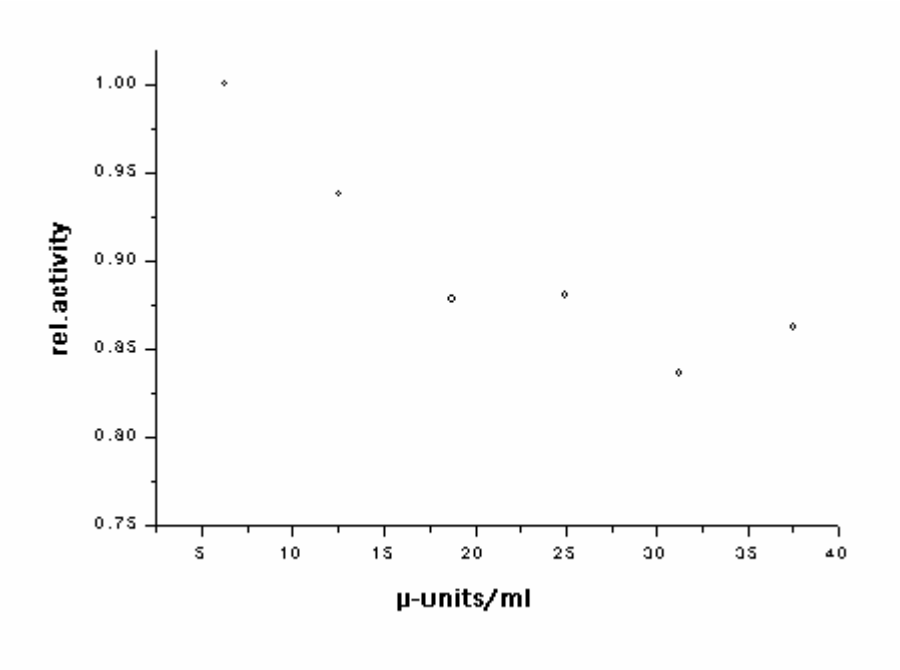


Fig 3.4.1.1: Relative activity of CPCR in water

In the microemulsion the effect is far bigger and cannot be ignored for either HLADH nor CPCR (Fig.3.4.1.2). Here the loss of relative activity amounted to $^2/_3$ over the observed concentration range. The explanation for this effect is a reversible aggregation of enzyme molecules forming oligomers. Whether this is true in the microemulsion too, where the limited size of the individual micelle would be expected to also limit the aggregation numbers is beyond the scope of this work.

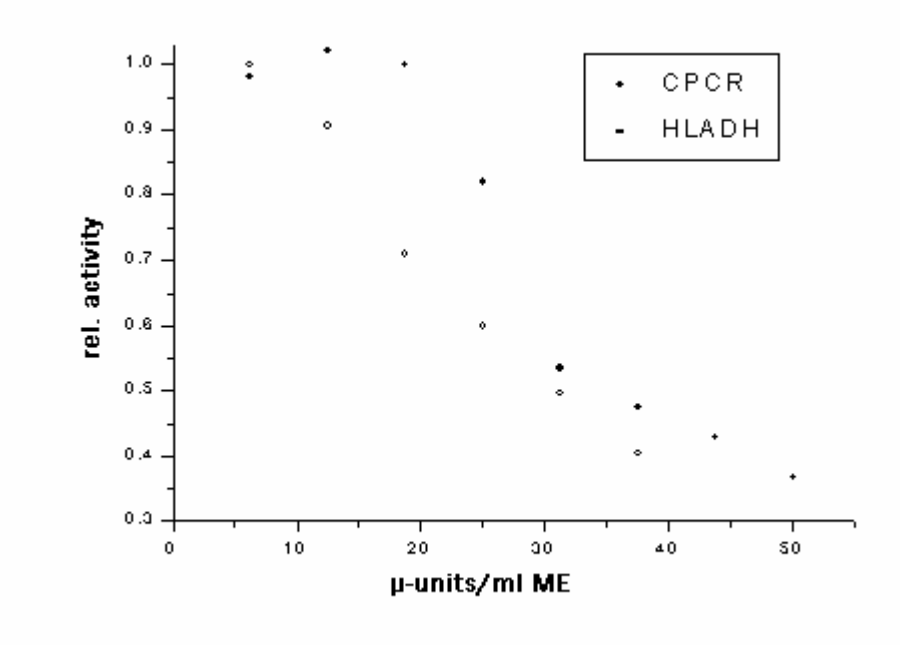


Fig 3.4.1.2 Relative activity of HLADH/CPCR in microemulsion

3.4.2 Enzyme activity and microemulsion composition

The usual environment for enzymes not bound to a carrier in technical processes is a homogeneous aqueous solution that is dominated by global factors like salinity, pH value and temperature. The physical characteristics of that solution are "normal", well-defined and open to direct measurement. A microemulsion (esp. a water-in-oil microemulsion) on the other hand is neither homogeneous nor are the physical characteristics of the aqueous phase necessarily "normal". Also the measurement of the standard parameters are difficult and have to rely more on indirect methods as has been shown in connection with the pH value (chapter 3.3). Even inside the individual micelle the microstructure of water changes depending on the distance from the surfactant layer. Near to the shell the characteristics deviate most while at the center conditions may be comparable to bulk water (provided the micelle is large enough) [Moulik 1998]. This naturally has an effect not only on the behaviour of pH indicators [El Seoud 1989] but also on that of enzymes. A variation of the two main parameters, γ and w_0 has a strong influence on the activity (and longevity) of the enzyme. For lipases the function of the enzyme itself depends on the presence of a polar/apolar interface (cf. chapter 1.5). They are therefore not just tolerant of the micellar environment but work best at low micelle diameters that force the enzyme unit into the interface layer. The behaviour of ADH is quite different (see below) and not yet completely understood. Fig.3.4.2.1+2 show the activity of HLADH and CPCR in dependence of the microemulsion parameters.

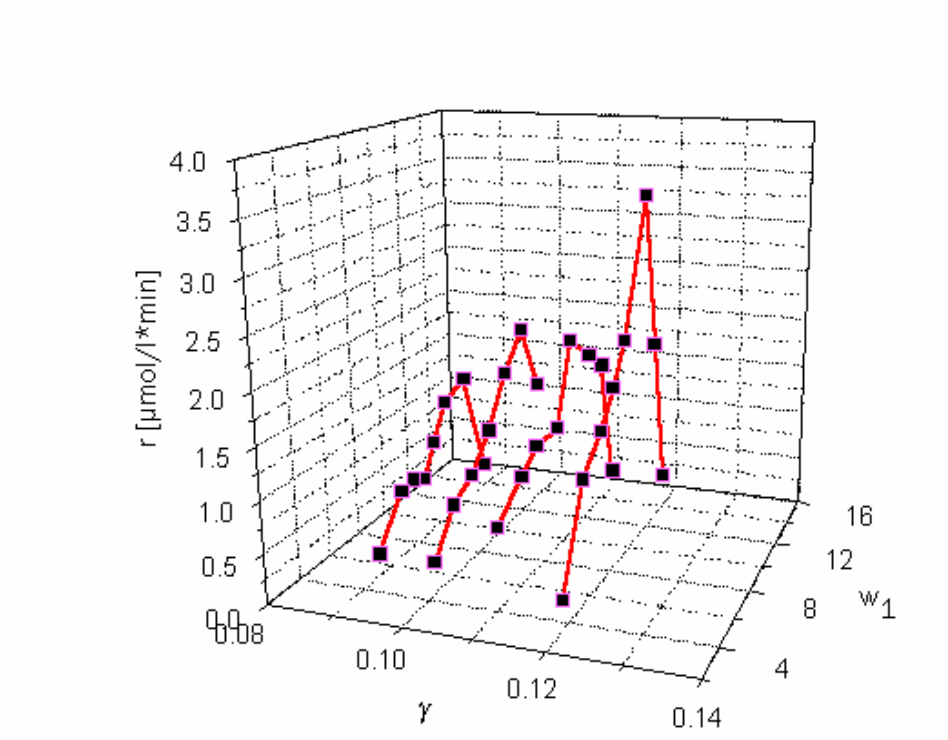


Fig.3.4.2.1: *HL*ADH (0,0375 mg/ml) catalyzed reduction of 2-butanone (0,67 mol/l) dependent on the microemulsion composition (γ / w_1) of the ternary mixture Marlipal O13-60 (detoppt) / water / cyclohexane) at pH 7 (c_{NADH} = 115 µmol/l).

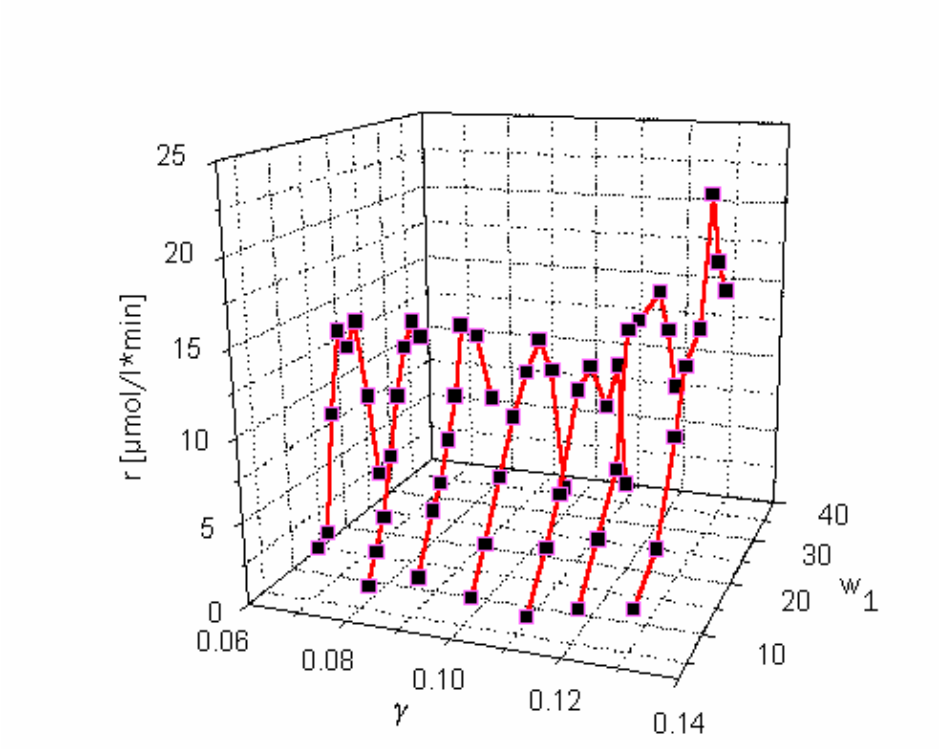


Fig.3.4.2.2: *CP*CR (0,13 mg/ml) catalyzed reduction of 2-butanone (0,67 mol/l) dependent on the microemulsion composition (γ / w_1) of the ternary mixture Marlipal O13-60 (detoppt) / water / cyclohexane) at pH 7 (c_{NADH} = 115 µmol/l).

Sections through the activity diagrams for constant γ and w_0 resp. show a characteristic behaviour as can be seen in Fig.3.4.2.3+4.

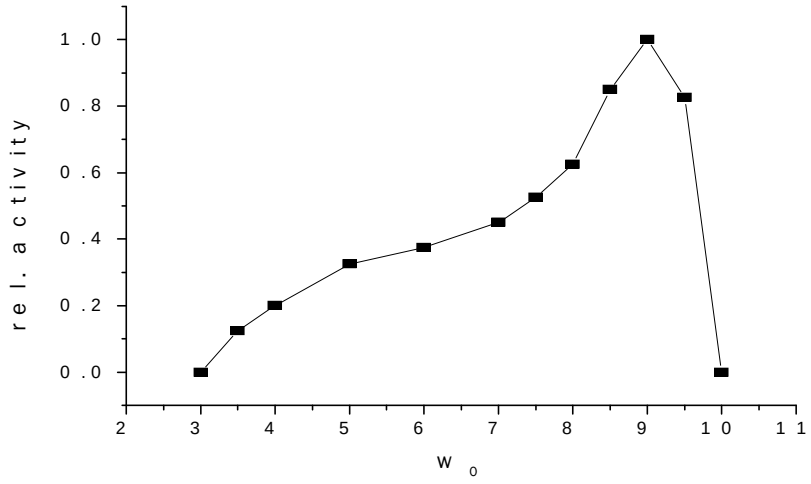


Fig.3.4.2.3: Relative activity of CPCR dependent on water/surfactant ratio (w_0) at constant surfactant concentration (γ=11) in a microemulsion of water/cyclohexane/Marlipal 013/60 (detopped)

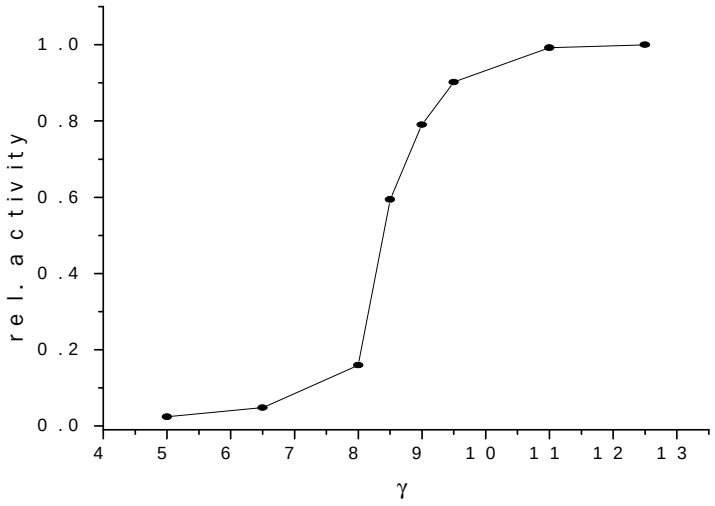


Fig.3.4.2.4: Relative activity of CPCR dependent on the surfactant concentration (γ) at constant water/surfactant ratio (w_0=7.5-8) in a microemulsion of water/cyclohexane/Marlipal 013/60 (detopped)

Appendix C shows the activity of ADH enzymes in microemulsions in a generalized way dependent on the parameters w_0 and γ. Incorporated are the data for *Horse Liver ADH* and *CPCR* in microemulsions made from cyclohexane, water and Marlipal 013/60 (older batch with high branching) as displayed in Fig.3.4.2.1+2. The main characteristic of the γ-dependency is the jump at a certain value of γ with practically no activity below and high activity above that extends to the boundary of stability of the 1-phase system. A mechanistic explanation for this is yet lacking. The cμc being far lower than the surfactant

concentration at the jump a yet unknown transition on the microscopic level has to be assumed but the available structural data don't show any abrupt change.

The w_0-dependency on the other hand is in accordance with the structural model of the reverse micelles. Up to the point where stable micelles are formed that can incorporate an enzyme molecule the enzyme activity is negligible because the ADH enzymes are usually inactive in an environment lacking a sufficient amount of water (even if no assumption is made about the influence of the solvent on the shape of the enzyme, water is necessary as provider of the second hydrogen added to the carbonyl function, the first being provided by the coenzyme). Once the water content is sufficient to stabilize reverse micelles, the ADH enzymes will be incorporated and begin to form a hydration shell competing for the water with the head groups of the surfactant. Thus a low but measurable activity can be observed that increases steadily with the increase in available water. This is the state of the so-called swollen micelle that contains an amount of water between zero and full hydration of the surfactant head groups but not yet a "pool" of free water. Once the saturation of the head groups is complete any further added water can be assumed to be used for the enzyme hydration and the formation of the "water pool". Given that 4-5 water molecules are needed for the full hydration of a surfactant molecule head group, the width of the sloped plateau seems to fit pretty well.

At this point the enzyme activity increases rapidly to its maximum. From here on it could be expected to decrease continually due to dilution and transport effects, i.e. decreased specific surface of the micelles and therefore diminished effective concentration of hydrophobic substrates at the location of the ADH enzyme that resides in the center of the "pool". With the available surfactants this effect cannot be observed because the capacity of the reverse micelles is exhausted at a value near the optimum of the enzyme. The phase transition towards a non-micellar state again reduces the enzyme activity to negligible values and also causes a swift and probably irreversible denaturation.

3.4.3 Enzyme deactivation in water-oil sytems

It was observed that enzymes seem to behave different in small volumes like a cuvette as compared to a larger vessel. The mixing conditions also have a measurable influence on the later enzyme activity. On a larger scale the mixing takes a significantly longer time, and the measured enzyme activity decreases in a rough correlation with it. It was also observed that the time at which the enzyme is added to the mixture plays a role. This led to the conclusion that either metastable phases occuring during the mixing process have

a detrimental effect on the enzyme or that any prolonged contact with the oil phase leads to the deactivation. It was now necessary to weigh these possibilities.

A series of experiments was therefore performed where ADH enzyme was brought in contact with mixtures of oil and water for defined periods of time.

CPCR was chosen as a model because it had, in the available preparations, a high initial activity. In comparison the HLADH and yADH were of low initial activity and had occasional solubility issues, i.e. parts of the lyophilisate would not readily dissolve. The enzyme was used dissolved in a 1:1 mixture of glycerol and water. Although no surfactant was used in these experiments, the small amount of enzyme solution had a significant emulsifying effect of its own. This is probably due to the protein nature of the enzyme preparation.

Direct injection of enzyme solution into a much larger oil phase (e.g. 100µl in 10 ml oil) followed by extraction with water (at least 4:1 water:oil) after varying times showed a measurable deactivation of enzyme, but it was too slow to sufficiently explain the strong, often complete loss of activity in large scale microemulsion formation tests. Injection through an oil phase into a large water phase showed no or only insignificant deactivation. In the case of a small amount of oil mixed with enzyme dissolved in large amounts of water the natural deactivation is slightly increased.

If the volumes of oil and water are similar, an exponential decay of enzyme activity can be observed (Fig.3.4.3.1). Under these conditions metastable phases (or aggregates) of water, oil and (probably) protein acting as surfactant occur but there are still distinct large domains of water and oil quickly separating once stirring or shaking is stopped. The nature of these aggregates is further discussed in chaper 3.12.

It is not clear, whether the decay follows the two-step model observed in microemulsions. The badly quantifiable sample extraction time is in a time range that can significantly distort measurements in the early stages of decay. A realistic offset of 10-30 seconds has an effect on the calculable kinetic constants that is so large that no meaningful values can be given. An offset of 20 s would imply a first order decay, a smaller one would require the assumption of either a second order decay or a more complicated sequential or parallel two-step model.

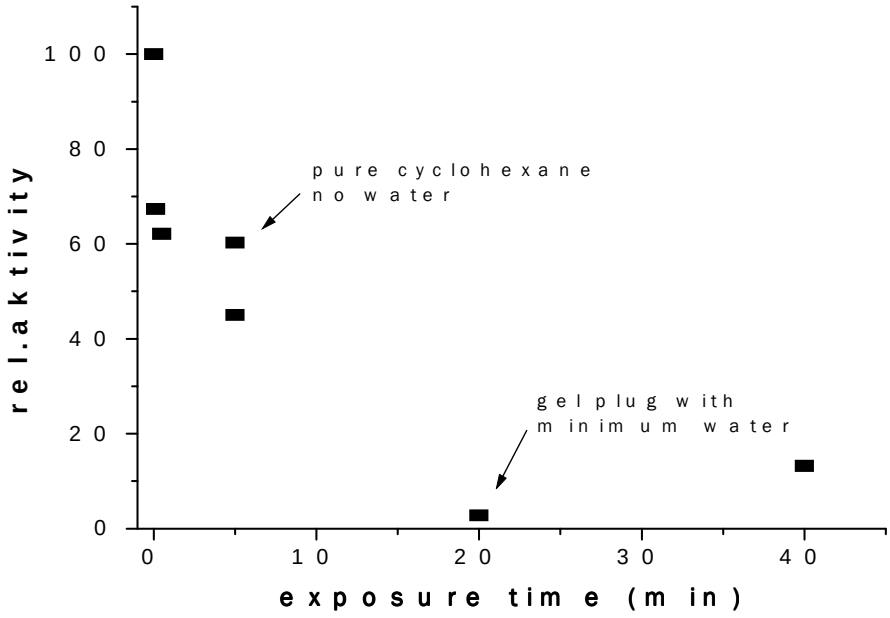


Fig.3.4.3.1: Enzyme (CPCR) deactivation in water/oil mixtures. Enzyme dissolved in 1:1 glycerol : water mixture. Near total deactivation in gel plug can be observed much earlier than the 20 minutes data point indicates.

If the fraction of water is reduced (i.e. more oil than water), the phase behaviour changes. The metastable phases (cf. chapter 3.12) become dominant, and at a ratio of water to oil of about 1:5 or lower almost no liquid phase can be observed anymore. The enzyme has to be extracted by adding large amounts of water in order to destroy the metastable phases. Under these circumstances the deactivation of the enzyme is complete before it can be removed for testing, that is in less than 2 minutes. Residual minimum activity could originate from enzyme remaining in the minimum of water not bound by the metastable phases.

If the ratio of water to oil is lower than about 1:20, the oil phase becomes dominant again and the metastable phases are formed in only insignificant amounts. The enzyme under these conditions shows deactivation behaviour similar to that in pure oil.

Comparision of enzyme taken out of the water phase in presence of metastable phases and extraction after dissolution of the metastable phases show a lower activity in the first case. That indicates that enzyme is absorbed in those phases, though not completely. If the metastable phases are removed (as opposed to dissolved), the enzyme deactivation in the remaining water phase is comparable to that in pure water.

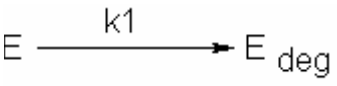
From this observation it can be concluded that the metastable phases absorb enzyme leading to fast deactivation while not influencing enzyme that is not entrapped in them. If the formation of these phases can be suppressed or their presence limited to a very short

time interval (<30 s), their strong detrimental effect can be reduced to acceptable levels. This problem and possible technical solutions are discussed in chapter 3.12.The presence of the oil phase on the other hand seems to have a minor effect that does not require special measures on the typical mixing time scales.

3.4.4 Enzyme activity with time and enzymatic decay

Enyzmes under working conditions decay with time, a process known as denaturation.
As shown [Berger 1999], the observed ADH activity change with time is dependent on the microemulsion composition and choice of surfactant. It can be mathematically described by basically 3 *formal* kinetic models (i.e. no claims about the microkinetic are made).

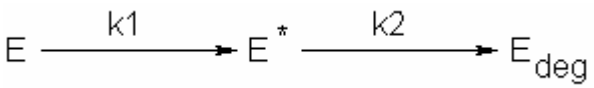
1. The decay is linear, i.e. zero order (see below) .
2. The activity change can be described as a one-step deactivation



following a first order kinetic. The activity is therefore described as:

$$I(t) = I_o * e^{-kt} \qquad \text{(eq.3.4.4.1).}$$

3. The study of ADH enzymes in microemulsions shows that this assumption does not suffice in describing the observed behaviour for all microemulsion compositions and has to be modified. An improved model assumes that the enzyme undergoes a transition into an excited state first before reaching the final inactive state. First order kinetics are again assumed.



If the specific activity of the original state and the excited state are set as i_1 and i_2 respectively, the total activity can be described as

$$I(t) = i_1 * e^{-k_1 t} + i_2 * \frac{k_1}{k_2 - k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right) \qquad \text{(eq.3.4.4.2)}$$

with i_1 equalling the I_0 of the one-step model.
This exact solution can theoretically not be used if the reaction rate constants are equal (or the difference smaller than the combined errors margins) but the function nonetheless converges to a finite value there. For practical means this is irrelevant because in that case the activity curves cannot be distinguished from those of a one-step model with just a different value of I_0.

If $i_2 > i_1$ the activity curve has a maximum at $t_{max} = \frac{\ln\left(1 + \frac{k_1}{k_2}\left(1 - \frac{i_1}{i_2}\right)\right)}{k_1 - k_2}$

By nonlinear regression the half-life constants for the individual steps ($t_{0.5} = {}^{1}/_{k} * \ln 2$) can be obtained.

With the tools available it is not possible to determine, whether the two- (or multi-) step model applies generally with a rate-determining step dominating in cases of seemingly one-step behaviour or whether different conditions change the actual mechanism.

For low values of γ CPCR shows an almost linear decay (and general low activity). For higher values of γ (and resulting higher activity) the decay shifts from 2-step at low w_0 values to apparent 1-step at high w_0 values. For values of both parameters approaching the stability boundary of the 1 phase microemulsion the data is inconclusive or the error margins too wide to properly distinguish between the different models.

3.4.5 Decay of Coenzyme

In addition to the enzyme the coenzyme NAD(P)H is the second component that significantly decays with time when brought into solution. For the reduced form the decay consists of (at least) two different processes, one reversible, the other(s) irreversible. In the reversible case the active functional group of the coenzyme is oxidized (loss of hydride ion), and the result is NAD(P)+. Thus the coenzyme is not lost but can be regenerated either enzymatically or by a hydride donor (this was tested successfully by addition of FDH or $NaBH_4$ resp). In a technical process that would simply mean that the amount of consumed regenerator substrate would exceed that of the intended product formed. In order to produce 1 mol of product S-alcohol in the ADH/FDH process it would be necessary to add more than 1 mol of formic acid. This reversible decay is not the dominant process in all cases though. In absence of enzyme the irreversible decay is responsible for at least ${}^{2}/_{3}$ of the measured decrease in the characteristic absorbance of NADPH (neither the oxidized nor the decomposed coenzyme absorbs in the area of 340 nm). Under the nearly neutral pH conditions required by the enzymes NADH is both more stable and decays mainly reversibly at a lower rate than NADPH. The decay can be considerably slowed down by lowering the temperature (Fig.3.4.5.1). The halflife of NADH is roughly tripled at 10°C compared to room temperature. The presence of purified surfactant seems to have only a minor effect (Fig.3.4.5.2) while some batches of raw surfactant accelerated the decay unpredictably.

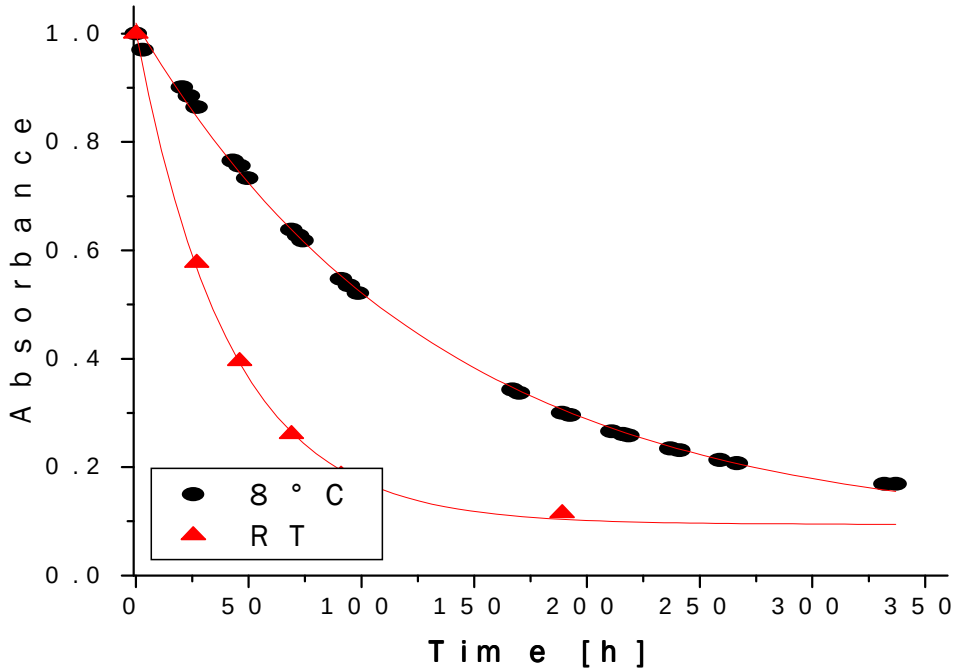


Fig.3.4.5.1 Decay of NADPH in aqueous solution at different temperatures

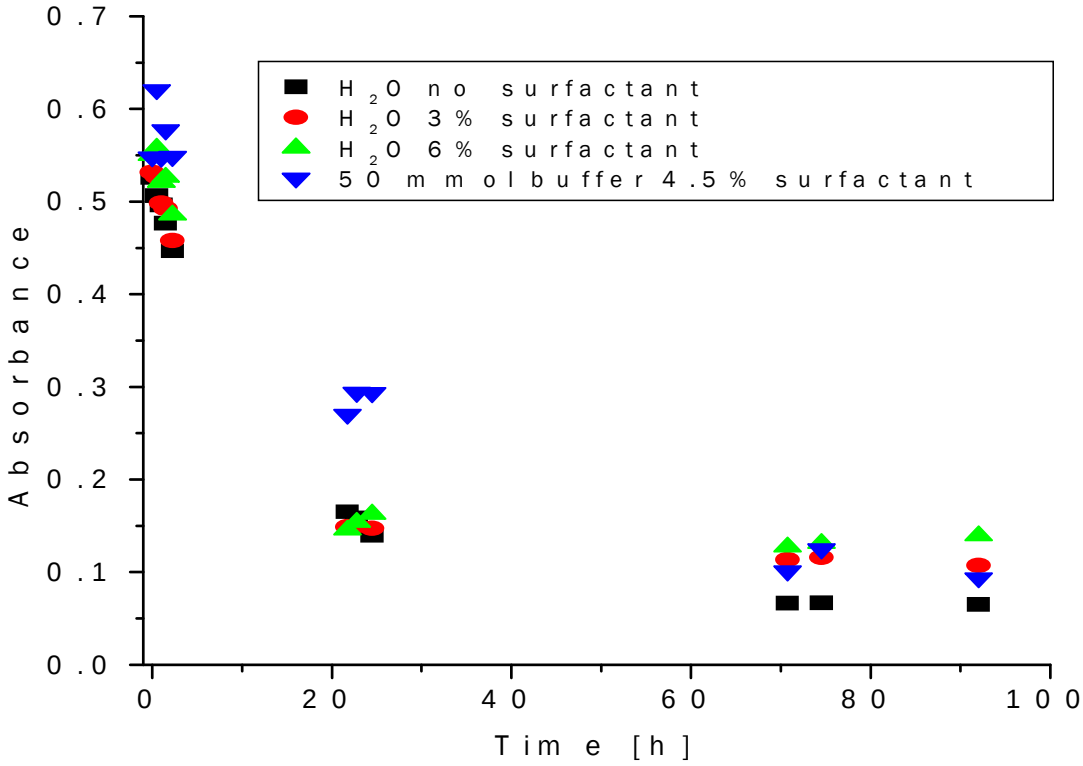


Fig 3.4.5.2: Decay of NADPH in aqueous solutions containing surfactant (purified Marlipal 013/60) and/or phosphate buffer

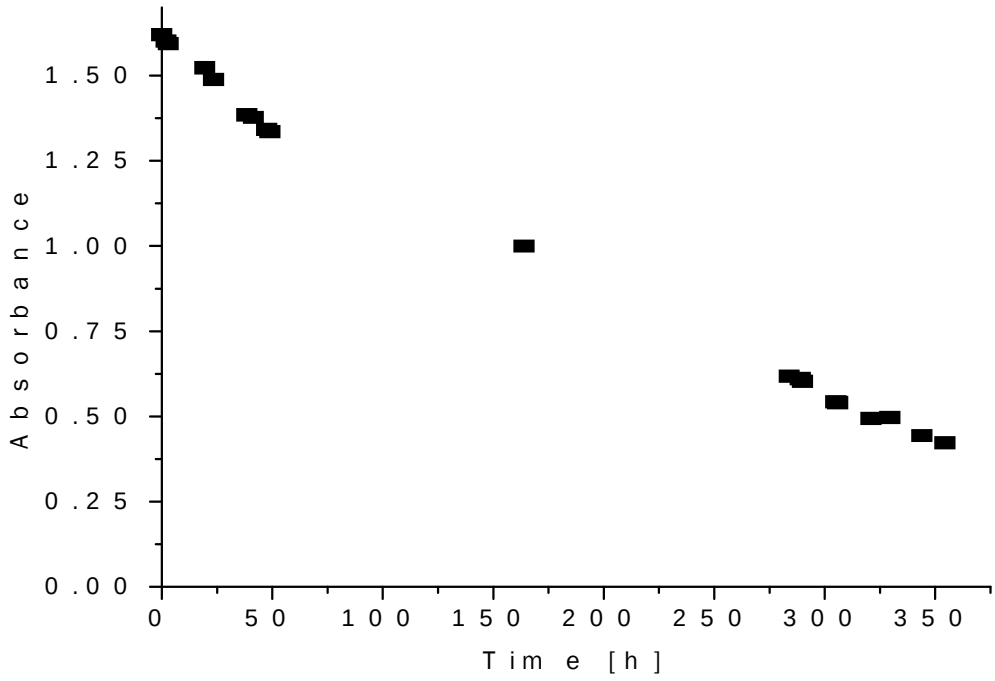


Fig 3.4.5.3 Decay of NADPH in a microemulsion (cyclohexane/Marlipal 013/60/water; γ=0.83, 1.2 w/w % water)

Interestingly the stability of NADPH seems to be slightly better in the microemulsion system than in a pure aqueous solution, and the curvature implies a lower reaction order (Fig.3.4.5.3). Another unexplained observation is that the curves seem to converge on a value higher than zero, although the absorbance of the decayed coenzyme is negligible. Samples quickly go to zero absorbance, when they are treated with heat or the pH value is changed to very high or low values, so it can't be an effect of the measurement conditions.

3.5 Concepts for a (semi)continuous process

The basic concept for the ADH/FDH process is a stirred tank reactor containing the w/o-microemulsion. Enzymes, cofactors and formate would be immobilized in the water droplets. Formic acid would be added as needed to maintain the formate concentration, at the same keeping the pH value constant. In case of a continuous process the reactor content would be pumped at a constant rate through a filtration loop that ideally rejects the micelles/water droplets while allowing the oil phase to pass. The reactor feed would consist of oil containing dissolved surfactant at a concentration equal to the cμc and also fresh ketone depending on the current conversion. Should the micelle retention be incomplete, the feed would also have to contain extra surfactant, water, formate, and

coenzyme. Enzyme retention has been complete in all experiments, i.e. no activity could be detected in the filtrate. Therefore only the decay of this component has to be considered. Fig.3.9.1 shows the basic flowsheet without the formate cycle.

The answer to the question, whether a continuous process is possible or useful, depends on two main factors, the stability of the enzyme and the quality of filtration. The shorter the half-life time of the biocatalyst, which cannot be replaced continuously, the more a batch process is the appropriate mode of operation. The quality of filtration mainly influences the cofactor. Since it is difficult to recover it down-stream, thus further complicating the product separation, cofactor passing the filter can be considered as lost and has to be replaced. Given the high cost this is of great influence on the economic viability. Should therefore either of those factors be unfavorable, the batch or semi-batch mode would be the only option.

In the following the main factors of influence are discussed for a simulation of the process. A general model for the filtration of microemulsions will be presented and its applicability discussed. A simplified version of this will be used in a process simulation based on the general reactor material balance of the ADH/FDH system. The results of this simulation will be used to decide which process (batch, semi-batch, CPCR) seems to be the most suitable. The results of this simulation will be compared to experiments.

3.6 Factors of influence (on a simulation)

The number of factors influencing the process is too big to be incorporated into a single simulation. For some of them and their interactions the available experimental data is insufficient, so approximations must be used. Highly complex models can be found in e.g. [Illanes 2003]. General thoughts on applicability e.g. in [Kister 2002].

Main parameters are:

- Halflife of enzymes and cofactors
- Activity of enzymes
- Quality of filtration (retention)

Main factors influencing those parameters:

- Microemulsion composition
- Temperature
- pH value
- concentration of diverse compounds
- mixing quality
- fouling resistance of membrane

Several of these factors have to be carefully balanced because they can influence each other positively as well as negatively.

Microemulsion composition

The main parameters for the microemulsion composition are the weight fraction of surfactant γ and the molar ratio of water to surfactant w_0 (w_1 for the micellar part alone). From experimental data (previously published by Berger [1999] and Orlich [2000(2)]), a general pattern can be deduced that can be easily fitted to a specific enzyme. Details can be found in Appendix C.

Temperature

Due to the negligible heat production per volume the temperature is the easiest to control but also has the strongest influence on the total process.

Temperatures above 20-25 °C exponentially accelerate the denaturation/decay of the enzymes and the cofactor. At 60 °C after only 20 minutes the ADH activity is down to a third of the initial value while the reaction velocity is not significantly increased. At 10 °C on the other hand the halflife of the cofactor is roughly tripled while the total reaction velocity is reduced (but still on a tolerable level). Lowering the temperature is therefore beneficial to the enzyme part of the process but unfortunately not to the microemulsion phase behaviour.

Depending on the average branching of the used surfactant there is an optimum temperature giving the maximum capacity for water in the w/o microemulsion. As the experimental data undeniably shows the ADH activity and longevity is highest near the maximum point (many micelles of large diameter). At this point the viscosity of the system begins to become critical. Any significant lowering of temperature increases the viscosity to intolerable values (mixture becomes solid at 4-8 °C depending on composition). At the same time the water capacity is reduced, so for a given composition a phase transition is to be expected. This can be either the formation of an excess phase or the internal transition from micellar to liquid crystalline. In both cases enzyme activity is lost (usually completely but theoretically reversible) and rapid denaturation occurs (irreversible).

A temperature between 10 °C and 20 °C would therefore be advisable sacrificing total velocity for vastly increased longevity while still retaining a tolerable fluid behaviour.

The latter is also of importance for the filtration. Fluids of higher viscosity are more difficult to filtrate and increase the probability of fouling. Concentration polarization is also increased due to the reduced diffusion especially near/in the laminary boundary layer.

pH value

Experimental data for the pH dependency of the used enzymes can be found e.g. in (Orlich Diss). The optimum performance interval for ADH/FDH is at near neutral values and pH deviation of more than 0.5 should be avoided. If the effect of the pH shift caused by the reaction and the compensating shift by the addition of formic acid are to be included as influences, the activity dependence might be approximated by a simple parabola (and not the more complicated Gaussian bell shape curve)

Concentration of diverse compounds

The main effect of dissolved compounds is on the phase behaviour of the microemulsion. Both salts and protein diminish the region of the stable 1-phase microemulsion. The capacity of water solubilization is reduced, thus limiting the maximum micelle size. Since for the ADH system large micelles are best for activity and stability, this reduction is likely to affect those negatively. Experimental data was not won on this.

Compounds dissolved in the oil phase can also influence the phase behaviour but are less critical here because the useful concentrations are relatively low. The strongest effect can be found with short-chain ketones and alcohols (iso-propanol, acetone). Those may on the other hand have a slight stabilizing effect on the ADH as experimental data suggests. The causality is not proven though.

Mixing quality and speed

This mainly influences the initial enzyme activity I_0. Assumptions about the degree of deactivation during mixing can be made on the basis of experimental data as described in chapter 3.4.4.

Fouling resistance of membrane

There are numerous mathematical models available in literature that describe this effect. The special problems involving microemulsions are described qualitatively in chapter 3.13.

3.7 Filtration of the Microemulsion, material balance

The description of the filtration has to take into account the two different states the surfactant is present in the system. A certain amount (cmc) is present monomolecularly dispersed (true solution), the rest forms micelles or reversed micelles (c_{mic}). The former will pass freely through the filter/membrane (permeation) together with the bulk solvent, the latter will be held back to a certain degree (retention R). Retention depends on the size and geometry of the micelles in relation to the used filter (cut-off, hydrophobicity etc.). The total surfactant concentration in the filtration system is given as

$c_{tot} = cmc + c_{mic}$ (eq.3.7.1)

Bulk solvent and surfactant enter the system with a volume flow $\dot{V}_{in}$ and a surfactant concentration of c_{in} resulting in a mass flow of

$\dot{m}_{in} = c_{in} * \dot{V}_{in}$ (eq.3.7.2)

Bulk solvent and surfactant leave the system (permeate) at a volume flow of $\dot{V}_{out}$.
The total concentration in the permeate consist of

$C_{perm,tot} = c_{out} = cmc + c_{perm,mic}$ (eq.3.7.3)

with

$c_{perm,mic} = (1\text{-}R)*(c_{tot} - cmc)$ (eq.3.7.4)

The permeate mass flow is therefore

$\dot{m}_{out} = (cmc + c_{perm,mic})* \dot{V}_{out} = [cmc + (1\text{-}R)*(c_{tot} - cmc)]* \dot{V}_{out}$ (eq.3.7.5)

(In case of $c_{tot} < cmc$ the permeate concentration is of course c_{tot} and the permeate mass flow is $c_{tot}*\dot{V}_{out}$). The total mass flow is therefore

$\dot{m}_{tot} = c_{in}*\dot{V}_{in} - [cmc*R + (1\text{-}R)*c_{tot}]* \dot{V}_{out}$ (eq.3.7.6)

As can be seen, in case of R=1 (total retention), the surfactant concentration in the permeate equals cmc and in the case of R=0 (no retention) it equals c_{tot}. c_{tot} is furthermore dependent on the relation of $\dot{V}_{in}$ and $\dot{V}_{out}$.

$c_{tot} = m_{tot} / V_{tot}(t)$ (eq.3.7.7)

with

$V_{tot}(t) = V(t) = V_0 + (\dot{V}_{in} - \dot{V}_{out})*t$ (eq.3.7.8)

The general mass balance can be given as ($m=m_{tot}$)

$$\frac{dm}{dt} = \dot{m}_{in} - R * cmc * \dot{V}_{out} - (1-R) * m * \frac{\dot{V}_{out}}{V(t)} \quad \text{(eq.3.7.9)}$$

or (modified)

$$\frac{dm}{dt} = c_{in} * \dot{V}_{in} - R * cmc * \dot{V}_{out} - \frac{(1-R) * m}{V_0 + (\dot{V}'_{in} - \dot{V}'_{out}) * t} * \dot{V}_{out} \quad \text{(eq.3.7.10)}$$

The case of total surfactant concentrations lower than cmc is neglected because it has no practical relevance to our filtration problem.

With $\dot{V}_{in}$ equal to $\dot{V}_{out}$, V_{tot} is constant (V_0) and solving the mass balance is simple (t is eliminated from the right hand side of the equation).

$$m(t) = E * m_0 + \frac{(1-E) * V_0 * (c_{in} - R * cmc)}{(1-R)} \quad \text{with} \quad E = \exp\left(-\frac{\dot{V}_{out}}{V_0} * (1-R) * t\right) \quad \text{(eq.3.7.11)}$$

Although a general analytical solution is possible, it is cumbersome and mathematically undefined (division by 0) at $\dot{V}_{in} = R * \dot{V}_{out}$ and $\dot{V}_{in} = \dot{V}_{out}$ (in this case the special solution for V=const has to be used)

$$m(t) = \frac{A * (C * V_0 - B * m_0) - C * V(t)}{B} \quad \text{(eq.3.7.12)}$$

with $A = \left(\frac{V_0}{V(t)}\right)^{\wedge}\left(\frac{(1-R) * \dot{V}_{out}}{\dot{V}_{in} - \dot{V}_{out}}\right)$; $B = \dot{V}_{in} - R * \dot{V}_{out}$; $C = c_{in} * \dot{V}_{in} - R * cmc * \dot{V}_{out}$

If the retention is lower than 100%, the loss of solubilized water has to be taken into account.

The water loss is coupled to the loss of micellar surfactant by the w_1 value.

$$w_1 = \frac{c_{H_2O}}{C_{surf}} * \frac{M_{surf}}{M_{H_2O}} \quad \text{(eq.3.7.13)}$$

If no water enters the sytem via $\dot{V}_{in}$, the mass balance for solubilized water is

$$\frac{dm_{H_2O}}{dt} = \frac{dm_{surf,mic}}{dt} * w_1 * \frac{M_{H_2O}}{M_{surf}}. \qquad \text{(eq.3.7.14)}$$

therefore

$$\frac{dm_{H_2O}}{dt} = -(1-R) * (c_{tot} - cmc) * \dot{V}_{out} * w_1 * \frac{M_{H_2O}}{M_{surf}} \qquad \text{(eq.3.7.15)}$$

This simplifies to

$$\frac{dm_{H_2O}}{dt} = -(1-R) * \dot{V}_{out} * c_{H_2O}. \qquad \text{(eq.3.7.16)}$$

If V = const (V_0), this is a simple first order kinetics.

$$m_{H_2O}(t) = m_{0,H_2O} * \exp(-\frac{(1-R) * \dot{V}_{out}}{V_0}) \qquad \text{(eq.3.7.17)}$$

Analogue equations can be given for substances dissolved in the solubilized water. The enzyme has a retention of 100% under all experimental conditions. Substrates dissolved in the continuous oil phase have normally a retention of 0%. At high pH values (alkaline) there can be a small extra loss of pH indicator via the oil phase because under these conditions the solubility in the oil is increased. Under normal conditions this can be ignored. If lost water is replaced via $\dot{V}_{in}$, it is recommended to solve the mass balance numerically or by coupling it to the surfactant mass balance.

This simplified model assumes the retention R to be a constant. It ignores that the size of the reversed micelles depends on both the actual surfactant concentration and the total concentration of solubilized water. The latter gives a fixed volume that has to be covered by surfactant, while the interfacial area is given by the former. The combination of both leads (barring a change in micellar geometry) to a fixed relation of micelle size und total number for a given volume of microemulsion. Under normal circumstances a size distribution can be neglected.

A decrease in surfactant concentration will decrease the number of micelles, while the individual micelle grows in size because the specific surface is in a reciprocal relationship to the radius.

For spherical micelles this is

$$a_s = \frac{A}{V} = \frac{4 * \pi * r^2}{4/3 * \pi * r^3} = \frac{3}{r} \qquad \text{(eq.3.7.18)}$$

At high values of R, i.e. low losses of solubilized water by micelles passing the filter, and no or insufficient external replacement of loss of monomerically dissolved surfactant, the micelles will grow and further increase the retention. At lower R values the contrary would be expected. This development is limited by the phase boundaries of the microemulsion system. With the surfactants used in this study, spherical micellar geometry can be assumed. At surfactant concentrations that would require to take into consideration deviant geometry without leaving the microemulsion domain, the macroscopic behaviour of the system tends to be unfavorable (e.g. too viscous).

In a dead-end filtration, i.e. with a diminishing total volume, the surfactant concentration increases. The total number of micelles grows but the individual micelle shrinks in size. This will lead to reduced values of R, i.e. a diminishing retention. The dependency of the rejection on the micelle size could be used to regulate the microemulsion composition over the surfactant feed. Two cases can be distinguished:

a) the surfactant feed concentration is lower than the cμc

b) the surfactant feed concentration is equal to or higher than the cμc

In the first case there is a loss of surfactant. This causes a transfer of surfactant molecules from the micellar towards molecularly dissolved state. As described this will cause the number of micelles to shrink and the diameter of the individual micelle to grow, thus also increasing the rejection.

In the second case the total interface increases causing the number of micelles to rise and the individual diameter to decrease, thus reducing the rejection. If the surfactant concentration drops below a certain limit, the micelles become unstable and a second phase will be formed.

If the stable domain of the microemulsion is not left, a new equilibrium between feed and permeation loss should be reached, i.e. the amount entering the system via the feed is equal to the loss by permeation. The increased loss of intramicellar content has of course to be considered too.

3.8 Applicability of the filtration model

The model as proposed in the preceding chapter makes several assumptions that might not accurately describe actually available membranes. It supposes that the filtration is a static process as far as the membrane itself is concerned and the flow and retention solely dependent on the membrane geometry and the composition of the solution to be filtrated. It also postulates, for mathematical simplicity's sake, a Gaussian or at least symmetrical pore size distribution. Tests by M.Schwarze [subm. 2007] indicate that the latter assumption is reasonably accurate for some commercially available membranes but not in general. Fig.3.8.1 shows the pore distribution for a selection of polymer membranes.

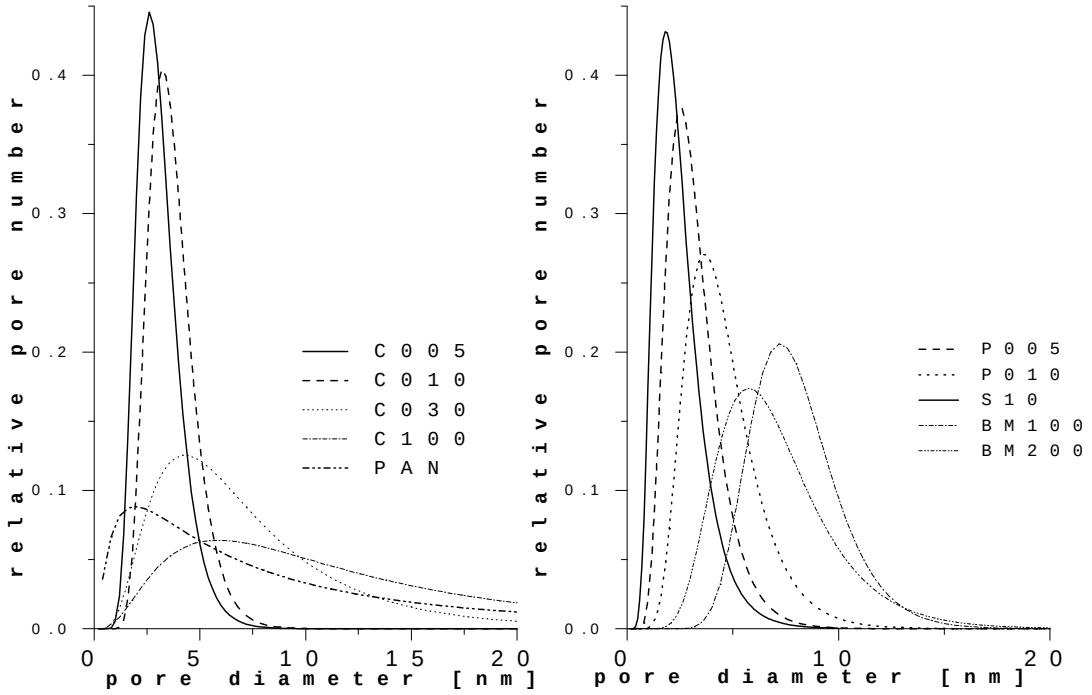


Fig. 3.8.1: Pore size distribution for commercially available polymer membranes (Nadir and Berghof) obtained through polymer filtration; courtesy of M. Schwarze, TU Berlin

It can be clearly seen that, while all samples show a tailing deviation towards larger pores, the degree can be significantly different. The C005, C010, S10 and P005 show a narrow distribution that reasonably approximates the Gaussian normal form. P010, BM100 and BM 200 are a good deal broader but the tailing is still within limits. All others cannot be accurately described with the normal distribution. It seems that those membranes with a low median pore diameter tend to have a more narrow distribution also. Given though the measured micelle diameters of about 3 nm maximum, none of the

depicted membranes would be suitable because the median pore diameter is in the same range or not much smaller even for the best. That would yield a roughly estimated retention of just about 50 to 66% while anything below 90% can be considered as insufficient.

3.9 Simplified process model and simulation

The simulation consists of two parts, the determination of the most suitable enzyme (with regard to activity and stability) and secondly the continuous process including cofactor regeneration under different assumptions for the achievable retentention of the cofactor. It does not consider the deactivation of the cofactor and also assumes that the composition of the microemulsion remains constant, i.e. the surfactant lost is exactly replaced through the feed. The kinetic data as obtained by Berger [1999], Orlich [2000(1,2)] and von Dziegielewski [2005] are used here. The software Berkeley Madonna® was used for the simulation. The specific programs for Batch and CSTR mode can be found in the appendix. Neither in the case of the Batch Reactor (BR) nor of the CSTR a separate heat balance is necessary because both the enzymatic reaction rates and the enzyme concentrations are low resulting in uncritical total heat flows.

In the BR there are no mass fluxes into or out of the reactor. The addition of formic acid for pH control and replacement of consumed formate is ignored here and the formate concentration assumed as constant. The terms for the enzymatic reaction are abbreviated as r_{ADH} and r_{FDH} for clarity's sake.

The balanced equations for the reactants are:

$$\frac{dc_{ketone}}{dt} = -r_{ADH}$$ for the educt ketone (eq. 3.9.1)

$$\frac{dc_{alcohol}}{dt} = r_{ADH}$$ for the product alcohol (eq. 3.9.2)

$$\frac{dc_{NADH}}{dt} = -r_{ADH} + r_{FDH}$$ for the reduced coenzyme form (eq. 3.9.3)

$$\frac{dc_{NAD+}}{dt} = r_{ADH} - r_{FDH}$$ for the oxidized coenzyme form (eq. 3.9.4)

For the enzymes a single-step or an oligo-step deactivation can be selected. Neglecting the exchange term and using the simplified form instead the resulting equation is:

$$\frac{dr_{\max,ADH/FDH}}{dt} = -r_{\max,ADH/FDH} * \sum_i A_i * k_{des,i} \text{ with } \sum_i A_i = 1 .$$ (eq. 3.9.5)

In the case of the CSTR with a filtration loop a distinction has to be made between components that do not pass the filter, those that do and those that do partially.
Enzymes do not pass the filter, oil soluble compounds are not affected by the filter, water soluble (i.e. micelle bound) compounds are partially rejected by the filter with the retention R. The surfactant is left out of the simplified balance here, the appropriate model has been described in chapter 1.8 and 3.7. For the enzymes the equation for the decay is used instead of that for the loss via filtration. A special case is the formate. It is consumed by the FDH, converted into gaseous CO2 and effectively regenerated by addition of formic acid. For the simplified model used here the formate concentration is assumed to be constant. The general flow diagram is shown in Fig. 3.9.1.

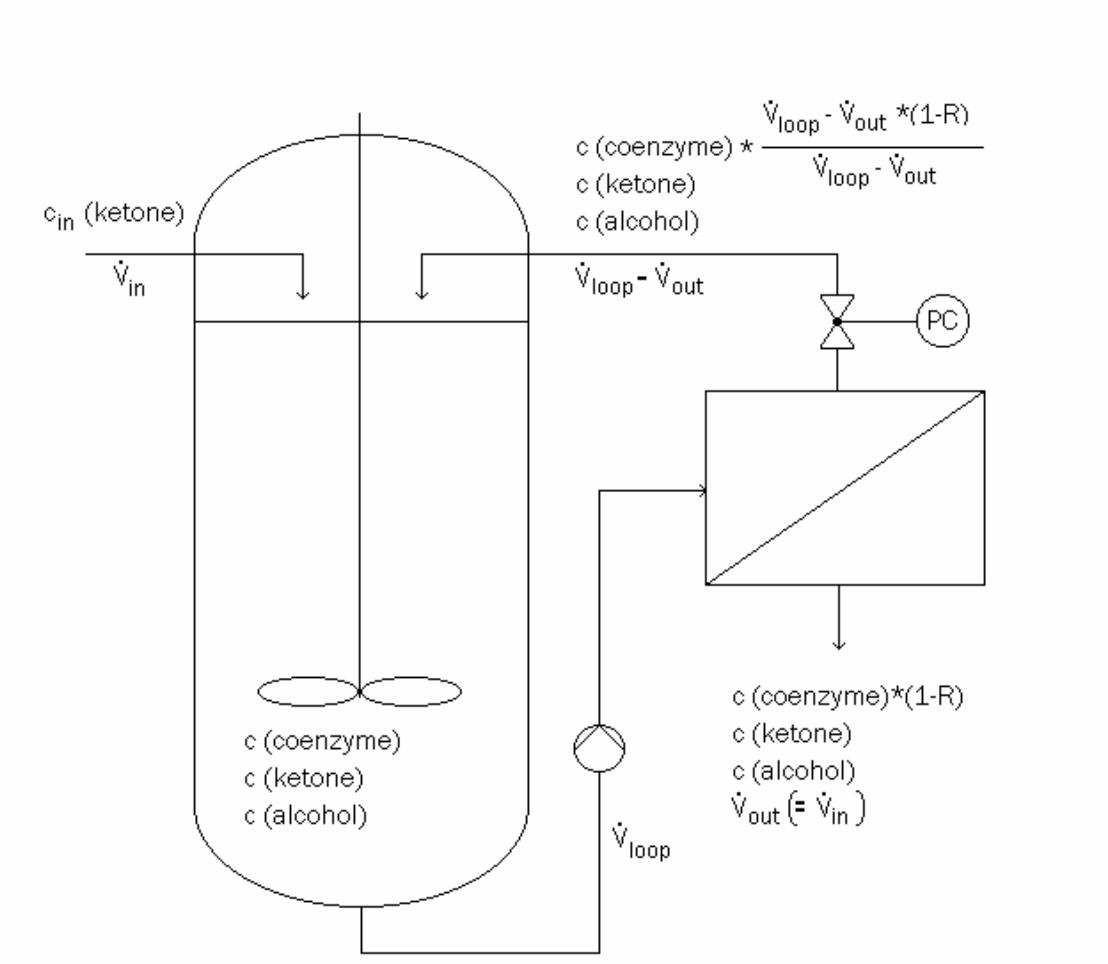


Fig. 3.9.1: Simplified flow diagram for simulated process. In case of batch mode all flows are zero. It is assumed that lost surfactant is replaced and that the formate concentration is constant.

Under the assumption that the loop is small in comparision to the total reactor volume and that the reaction volumes stays constant, a separate balance is not necessary, and the flows in and out of the reactor can be expressed by the residence time τ. The index 'in' refers to the flow into the reactor, unindexed variables refer to the reactor content.

The resulting material balanced equations for the reactants are:

$$\frac{dc_{ketone}}{dt} = \frac{1}{\tau} * (c_{ketone,in} - c_{ketone}) - r_{ADH}$$ for the reactant ketone (eq. 3.9.6)

$$\frac{dc_{alcohol}}{dt} = -\frac{1}{\tau} * c_{alcohol} + r_{ADH}$$ for the product alcohol (eq. 3.9.7)

$$\frac{dc_{NADH}}{dt} = \frac{1}{\tau} * (c_{NADH,in} - c_{NADH} * (1-R)) - r_{ADH} + r_{FDH}$$ for the red. coenzyme (eq. 3.9.8)

$$\frac{dc_{NAD+}}{dt} = -\frac{1}{\tau} * (c_{NAD+} * (1-R)) + r_{ADH} - r_{FDH}$$ for the ox. coenzyme (eq. 3.9.9)

For the enzymes a single-step or an oligo-step deactivation can be selected. Neglecting the exchange term and using the simplified form instead the resulting equation is:

$$\frac{dr_{\max,ADH/FDH}}{dt} = -r_{\max,ADH/FDH} * \sum_i A_i * k_{des,i} \quad \text{with} \quad \sum_i A_i = 1 .$$ (eq. 3.9.10)

It is assumed here that lost coenzyme is replaced in the NADH form (if at all) and that the natural decay of coenzyme is negligible.

The rate law for the *alcohol dehydrogenase* is the Michaelis-Menten equation [1913] modified for two substrates.

$$r_{ADH} = r_{\max} \cdot \frac{[NADH]}{K_{M,NADH} + [NADH]} \cdot \frac{[Ketone]}{K_{M,Ketone}\left(1 + \frac{[Ketone]}{K_{i,Ketone}}\right) + [Ketone]}$$ (eq. 3.9.11)

The *formate dehydrogenase* follows the same pattern (eq. 3.9.12).

$$r_{FDH} = r_{\max} \cdot \frac{[Formate]}{K_{M,Formate} + [Formate]} \cdot \frac{[NAD^+]}{K_{M,NAD^+}\left(1 + \frac{[NADH]}{K_{i,NADH}}\right) + [NAD^+]}$$

The deactivation can be described either through the 1- or the 2-step model.

$$\frac{dr_{\max,ADH}}{dt} = -k_{des} \cdot r_{ADH,0}$$ (eq. 3.9.13)

$$\frac{dr_{\max,CPCR}}{dt} = -[A_1 * k_{des,1} * r_{CPCR,0} + A_2 * k_{des,2} * r_{CPCR,0}]$$ (eq. 3.9.14)

The kinetic data appertain to pure water or a microemulsion with γ=10% and w_0=5 resp. The data has been derived from measurements by Orlich, Berger and von Dziegielewski over a longer time period using different batches of enzyme and surfactant. The given values may therefore differ slightly from any set published previously.

Tab. 3.9.1: Kinetic data for the cofactor regeneration by FDH

FDH	Water	Microemulsion
r_{max}	12,65 µmol/(min mg)	0,32 µmol/(min mg)
$K_{M,NAD+}$	84 µmol/l	130 µmol/l
$K_{M,Formate}$	54 mmol/l	200 mmol/l
$K_{i,NADH}$	35 µmol/l	50 µmol/l

Tab. 3.9.2: Kinetic data for ADH from yeast

yADH	Water	Microemulsion
r_{max}	0,053 µmol/(min mg)	0,65 µmol/(min mg)
$K_{M,2\text{-Heptanone}}$	6,36 mmol/l	612 mmol/l
$K_{M,NADH}$	20 µmol/l	210 µmol/l
$K_{i,\pm 2\text{-Heptanol}}$	26 mmol/l	>1000 mmol/l

Tab. 3.9.3: Kinetic data for CPCR in microemulsion

Parameter	Value
$K_{M,NADH}$	$1,0 \pm 0,1$ µmol/l
$r_{max,2\text{-Butanone}}$	$0,026 \pm 0,003$ U/mg
$K_{M,2\text{-Butanone}}$	100 ± 40 µmol/l
$r_{max,Acetone}$	$0,076 \pm 0,003$ U/mg
$r_{max,Acetophenone}$	$0,047 \pm 0,002$ U/mg
$r_{max,2\text{-Octanone}}$	$0,052 \pm 0,002$ U/mg
$K_{M,2\text{-Octanone}}$	21 ± 5 µmol/l
$k_{des,1}$	$0,249 \pm 0,026$ d^{-1}
$k_{des,2}$	$4,32 \pm 1,30$ d^{-1}
A_1	$0,63 \pm 0,04$
A_2	$0,33 \pm 0,04$

Tab. 3.9.4: Kinetic data for HLADH

Parameter	Value
$K_{M,NADH}$	6 ± 1 µmol/l
$r_{max,2\text{-Butanone}}$	$(23 \pm 3)\ 10^{-3}$ U/mg
$K_{M,2\text{-Butanone}}$	< 0,1 µmol/l
$r_{max,Acetone}$	$(50 \pm 5)\ 10^{-3}$ U/mg
$r_{max,2\text{-Octanone}}$	$(6 \pm 1)\ 10^{-3}$ U/mg
$r_{max,Acetophenone}$	$(10 \pm 1)\ 10^{-4}$ U/mg
$r_{max,i\text{-Butylmethylketone}}$	$(8 \pm 2)\ 10^{-3}$ U/mg
$K_{M,i\text{-Butylmethylketone}}$	73 ± 35 mmol/l
$k_{des,1} = k_{des,2}$	stable (< 10^{-2} d^{-1})

For the determination of the appropriate enzyme the substrate concentration was varied in the simulation to achieve a conversion > 80%. The enzyme concentration was set to 130 mg/l in relation to the aqueous phase. The cofactor was set to 100 µmol/l for NADH and 30 µmol/l for NAD+. The initial concentration for sodium formate is 500 mmol/l. The assumed reactor configuration is batch.

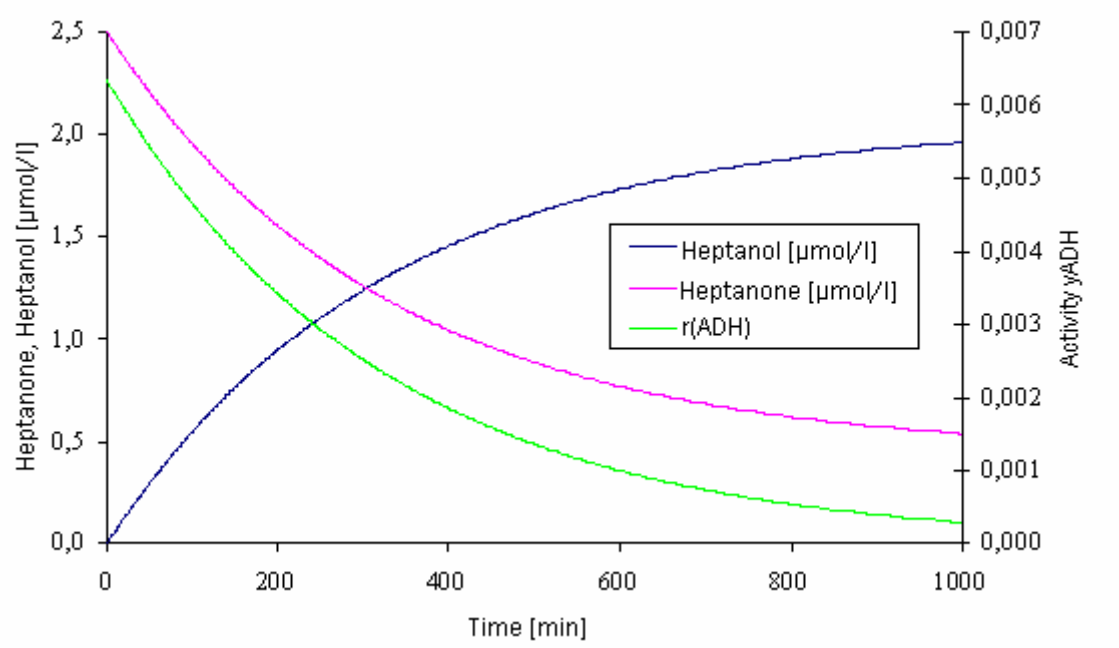


Fig. 3.9.2: yADH in a Batch reactor

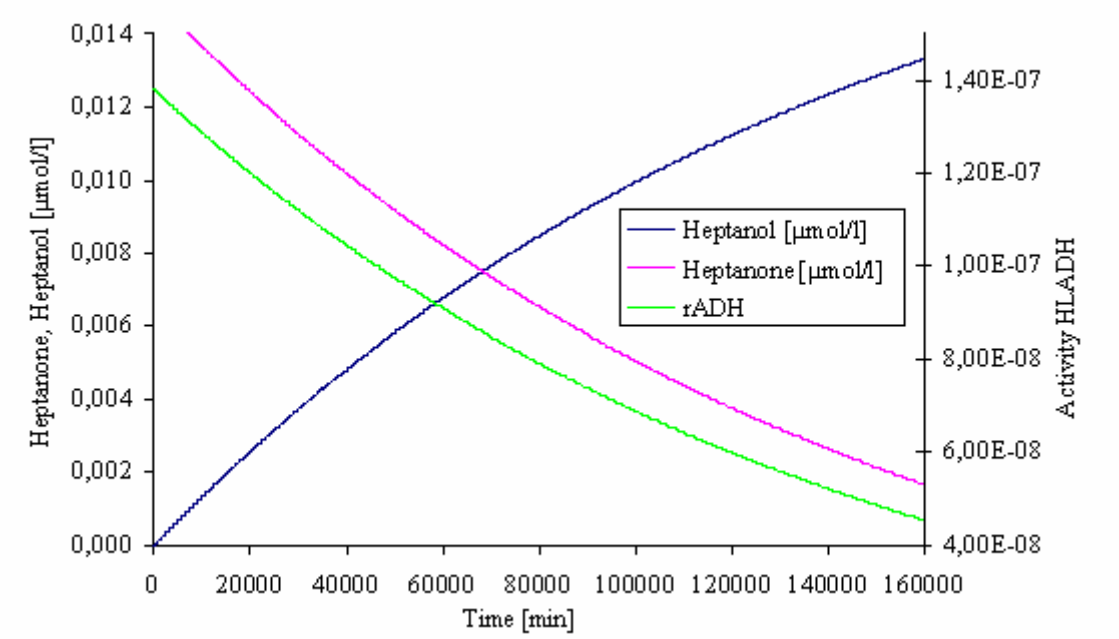


Fig. 3.9.3: HLADH in a Batch reactor

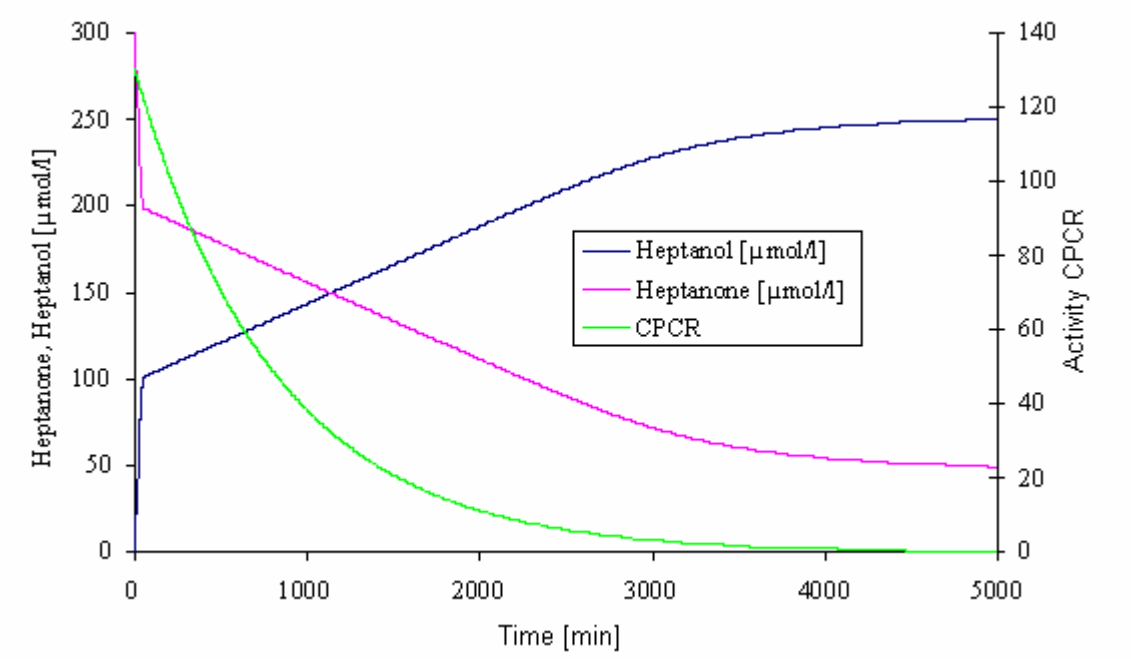


Fig. 3.9.4: CPCR in a Batch reactor

As can be seen yADH (Fig. 3.9.2) shows good activity but only a very low stability in the microemulsion, HLADH (Fig. 3.9.3) a high stability but much lower activity, and CPCR (Fig. 3.9.2) a very high activity while being less stable than HLADH. The jump in the curve in the case of CPCR is the result of the cofactor regeneration becoming the rate determining step. The initial amount of cofactor being used up the reaction is then limited by the FDH. As can be seen the reaction rate remains almost constant despite the deactivation of the CPCR. After about 50 hours the deactivation shows its effects and the rate determining process switches from the FDH to the CPCR. The NADH concentration in the reactor can be seen in Fig. 3.9.5. In the case of the other ADH enzymes the activity was either low enough or the deactivation fast enough to make them rate determining. A process using CPCR would require a much higher FDH concentration as a counterbalance. Otherwise the potential of the CPCR is wasted. The observed deactivation seems to be more or less independent of the "workload" of the enzyme (exception: slightly increased decay at total absence of substrate/coenzyme), so it should be kept at substrate concentrations where the reaction rate is highest.

In the following figures (3.9.6-8) the simulated results for a CSTR reactor with a cofactor retention of 100%, 98%, and 95% resp. can be seen. The enzyme deactivation follows the same kinetics as for the batch mode above.

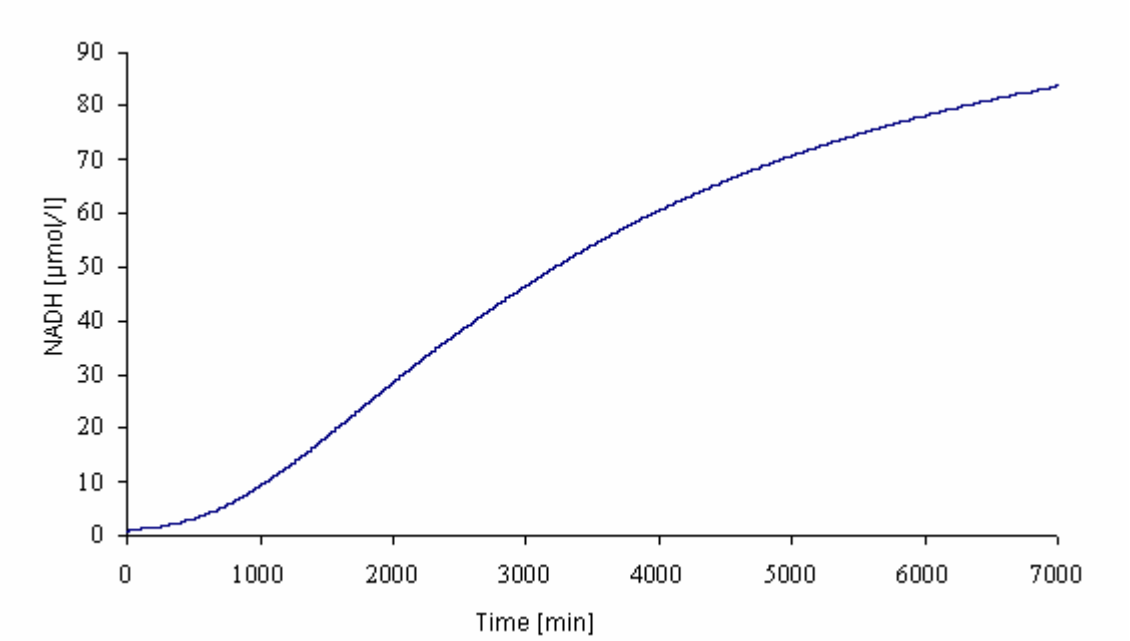


Fig. 3.9.5: NADH concentration for the simulated CPCR-FDH process

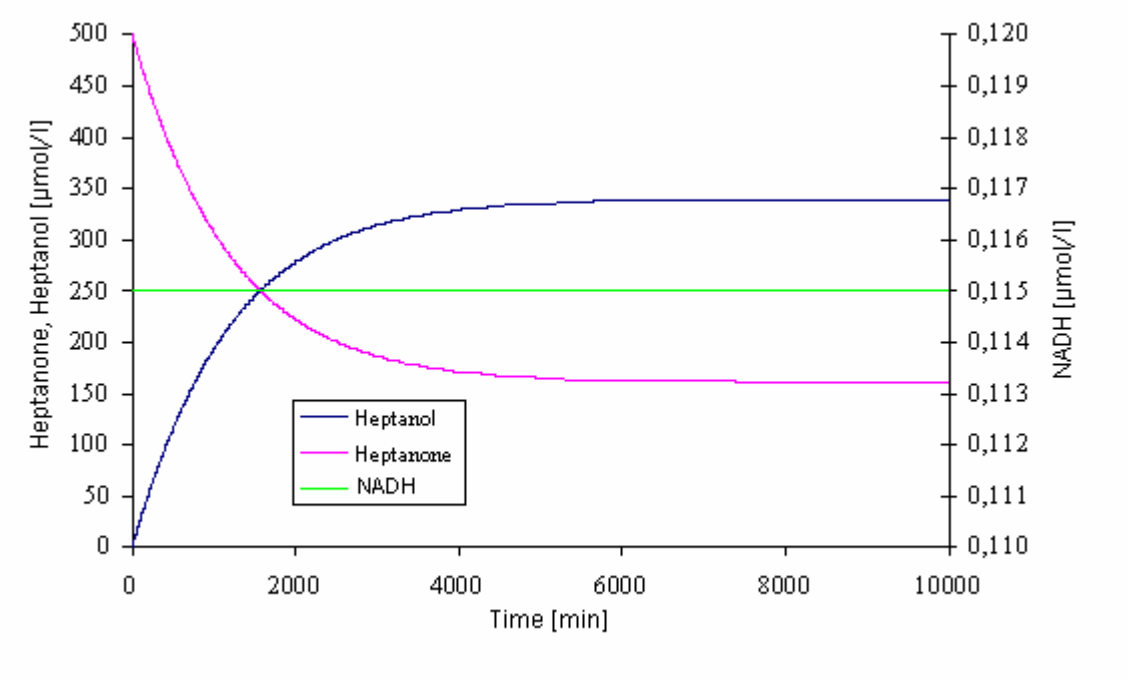


Fig. 3.9.6: CPCR/FDH in a CSTR with 100% cofactor retention, simulation time about one week

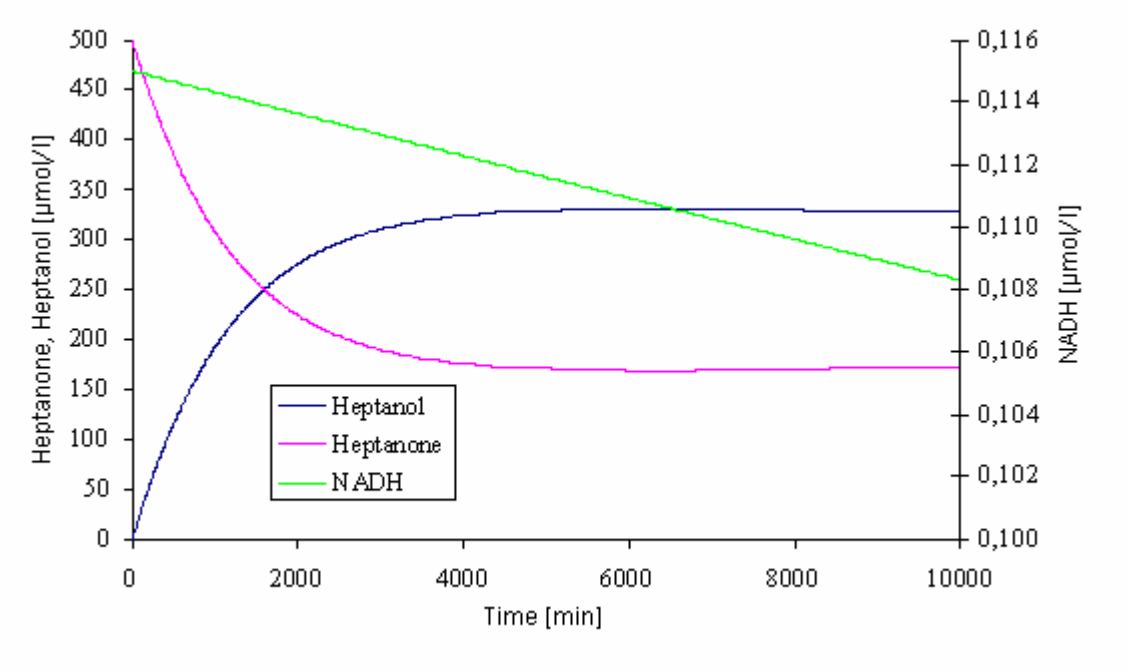


Fig. 3.9.7: CPCR/FDH in a CSTR with 98% cofactor retention, simulation time about one week

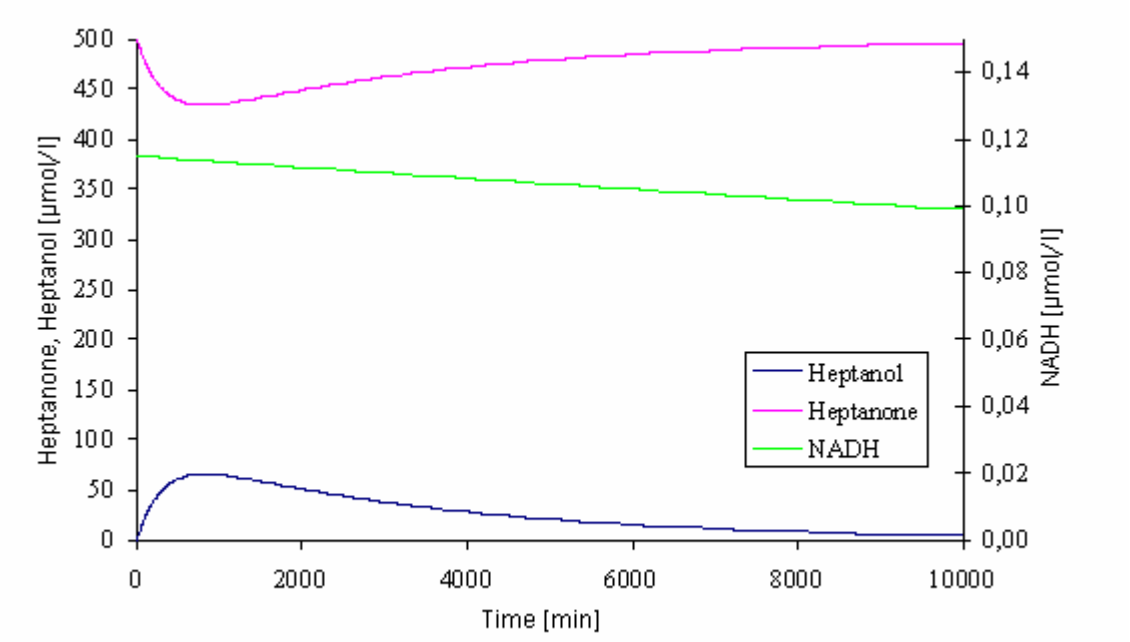


Fig. 3.9.8: CPCR/FDH in a CSTR with 95% cofactor retention, simulation time about one week

The simulation unambiguously shows that even at 95% retention of the cofactor the achievable conversion is insufficient. Given the practical experience with available ultrafiltration membranes, an economically viable continuous process seems unrealistic. And this even leaves out the question of cofactor decay. In any case the residence time necessary for an acceptable conversion is very high (in the range of days). Therefore only a batch process can be considered under the given circumstances.

Simulations for batch processes yielded 2.5 mmol/l 2-heptanol for an initial concentration of 3 mmol/l of 2-heptanone, i.e. a conversion of about 83%. Doubling that concentration leads to an increased yield of 2.8 mmol/l while the conversion drops to just 47%. Higher concentrations may be nonetheless preferable because of the higher saturation of the enzyme. As has been said above, this may have a stabilizing effect but it has to be balanced against possible inhibition by either educt or product and the destabilizing effect on the microemulsion.

3.10 Attempts at upscaling the ADH/FDH process

Three consecutive attempts were made to upscale the coupled ADH/FDH from the cuvette level to that of a standard lab reactor (reaction volume about 400 ml) using the data won from the simulation.

Table 3.10.1.: Initial chemical composition of reactor contents

Component	1st Attempt	2nd Attempt	3rd Attempt
NADH [g]	0.0287	0.0282	0.0292
NAD+ [g]	0.0085	0.0080	0.0083
FDH (8 U/ml) [g]	0.0572	0.0583	0.0773
CPCR (14.5 U/ml) [g]	0.0542	0.0574	0.0775
Sodium Formate [g]	0.0155	0.0463	0.1512
Heptanone [g]	0.0137	0.2452	0.2552
Caustic Soda (pH 12.7) [ml]	4	4	3.5
Dist. Water (boiled) [ml]	6	6	5.5
Cyclohexane [ml]	351	351	325
Marlipal 013/50 [ml]	39	39	36

The reactor was sterilized before each experiment by rinsing it thrice with boiling water, then once with methanol and drying it over night at 70°C in the drying cabinet. First the microemulsion was prepared and neutralized with the caustic soda before the active components were added. The pH value was checked using bromocresol purple.

1st Attempt

The active components were added undiluted under moderate stirring and the vessels containing them rinsed with reactor content thrice to ensure that the addition is complete. No enzyme activity was detectable and no product could be found when the reactor content was analyzed by gas chromatography.

2nd Attempt

The reactor containing the microemulsion was stirred for 20 minutes before the active components were added. These were dissolved in 0.5 ml demineralized water each before adding them to the stirred microemulsion. Samples were withdrawn and treated with concentrated formic acid to bring the reaction to a halt (by quickly decomposing the coenzyme and movinng out of the activity window of the enzymes) before freezing them for storage. Before analysis the samples were additionally heated to 65°C for 20 minutes. Only minimal pH changes were measured during the experiment and the product concentration was to low to be measured with the available equipment.

3rd Attempt

The reactor was stirred over night after neutralisation of the microemulsion. The active components were added dissolved in distilled water. Unlike in the first two attempts the addition was carried out stepwise waiting for one component to be completely dissolved before the next followed. The enzymes were added last. The change in the pH value was also low in this experiment but enough product was formed (total yield ~ 1%) for a quantitative analysis (Fig.3.10.1). There is a general correspondence with the simulated curves but the total activity is far lower and the deactivation much faster than predicted.

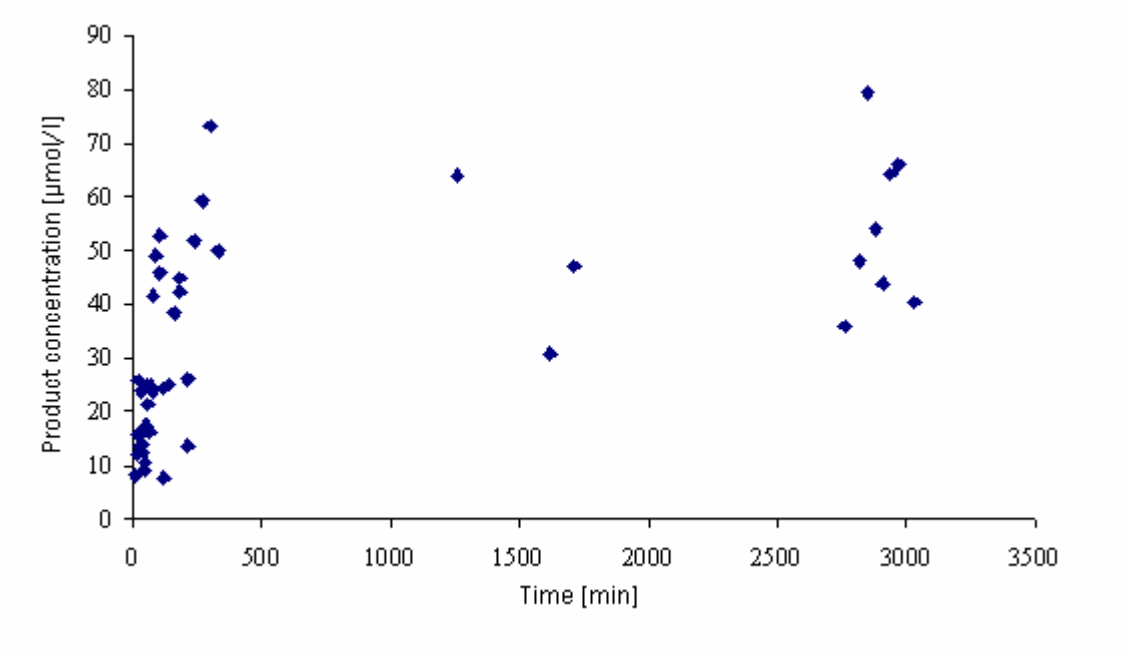


Fig 3. 10.1: Product formation with time in the 3rd upscaling attempt. Yield about 1%.

The most likely explanation is the deactivation of the enzyme during the time necessary for the dissolution and formation of the microemulsion, corroborated by the mixing experiments described in chapter 3.12.

3.11 Product separation

The selectivity of the investigated ADH enzymes is so high that no second product could be found. The task is therefore just to remove the product from the microemulsion. Since the enzymes can be securely retained by filtration, the separation is reduced to a mixture of product, residual educt, cyclohexane, surfactant, and small amounts of water. Temperatures too high or great shifts in pH value have to be avoided to prevent racemisation of the product alcohol. The surfactant is the main problem here because of its foaming, especially under the conditions of vacuum distillation (necessary to allow for relatively mild temperatures). It seems also to have a tendency to "hold fast" to polar components, thus impeding and prolonging the process.

It is also impossible to freeze out components because the microemulsion/surfactant rich phase solidifies as a whole. It is possible break the emuslion and to enrich the organic phase in product (and residual educt) while depleting it in surfactant by a multi-step sequence of temperature switches, alternating addition of organic solvent and water or brine followed by centrifugation (to accelerate the phase separation). Residual educt and product can be separated either chromatographically or by removing the ketone through precipitation (e.g. with sulfite or semicarbazide chloride) [Kruse 1995/1996]. Any addition of course prevents a simple recycling of components as originally envisaged.

3.12 Formation of microemulsions on different size scales

Observations by von Dziegielewski [2005] showed that the mixing time of the microemulsions is critical for the enzyme activity (cf. chapter 3.4.3). An activity loss of 50% or more in just 30 seconds requires a mixing time of less than 10 seconds. This is easily achieved on the cuvette level by shaking, for any medium or large scale process it represents a formidable problem. Mixing enhancement by e.g. ultrasonication is not an option here because it is known to have a deleterious effect on the enzyme.

The only practical way that was found is the premixing of surfactant and oil (cyclohexane) and adding the water to be solubilized afterwards under conditions as described below. If possible, the enzyme fraction should be added last because an existing microemulsion usually absorbs added dispersed phase more easily than one not yet formed. But this effect decreases significantly at the upper capacity limit that unfortunately is also the least unfavorable composition for the enzymatic process.

The macrodispersion of water in the oil/surfactant mixture is fast even on a larger scale (1-2 seconds for a 3 ltr. batch at only medium stirrer speed). The critical part is the transition from the macro- to the micro-dispersed state. If the wrong conditions are

selected, the formation of a metastable gel-like intermediate phase is favored. Enzyme that is trapped in this phase is deactivated almost instantaneously and is thereby lost. This phase also tends to form an extremly sticky layer at surfaces where the shear flow is lowered for whatever reason (baffles or the reactor walls, if the stirrer is slightly eccentric). Once formed, this layer can't be removed again by increased shear or even scrubbing but only dissolved by excess water after emptying the reactor. The composition of this intermediate phase (see Table 3.12.1) hints towards a lamellar phase, though a thorough analysis was not done. By entrapping a large percentage of the water and only slow transition to the thermodynamically favored microemulsiom state, the mixing is slows down in an unacceptable way. The suppression of the formation of the intermediate phase is therefore absolutely critical.

Tab. 3.12.1: Composition of metastable phases from cyclohexane/water/surfactant (LE7) Comparision of a fresh sample with one allowed to "age" for a few hours.

Compound	fresh sample	aged sample
Surfactant	17.5 wt.-%	22.4 wt.-%
Water	23.5 wt.-%	30.2 wt.-%
Cyclohexane	59 wt.-%	47.3 wt.-%
Physical Characteristics	opaque, very sticky, highly resistant to shear flows	partially transparent, less sticky, can be washed off by moderate shear flows

Mixing experiments were carried out over a range from about 50 ml to 3 ltr. in cylindrical beakers. At least at lower volumes the differences were insignificant. A pitched blade stirrer at a speed of 250-2000 rpm was used for most experiments, other stirrer types of different sizes mainly for qualitative comparision. Between 1 and 4 baffles were installed depending on volume and stirrer size/speed. The mixing was observed visually. The tendency of the intermediate phase mentioned above to stick to surfaces in absence of strong shear currents would have made the use of an optical probe impractical in any case.

In general the mixing in a stirred system proceeds in clearly distinguishable stages. First, in about 1-2 seconds, a homogenous opaque macrodispersion is created. This macrodispersion begins to segregate into gel-like flakes in a turbid solution. The turbid solutions begins to clear provided that the amount of added water does not exceed the capacity of the microemulsion. At the same time the flakes begin to dissolve. At this stage the outcome depends on the power (derived from speed and size) and position of the stirrer.

The density of the flakes is higher than that of the surrounding liquid, they tend to aggregate at the bottom of the vessel. If the stirrer is installed at to high a position and/or running at too low a speed, these dregs will not dissolve at all. These dregs are not identical with the intermediate phase but have a lower content of oil and a slightly higher of surfactant. They are not sticky and partially translucent as opposed to the completely opaque intermediate phase. At sufficient stirrer speed the flakes are dissolved completely leaving either a clear or, if the capacity is exceeded, slightly turbid but homogenous solution (liquid crystals?). In all these experiments the water to be solubilized was added in one single portion. Fig.3.12.1 shows the dependence of mixing time on total volume and stirrer speed.

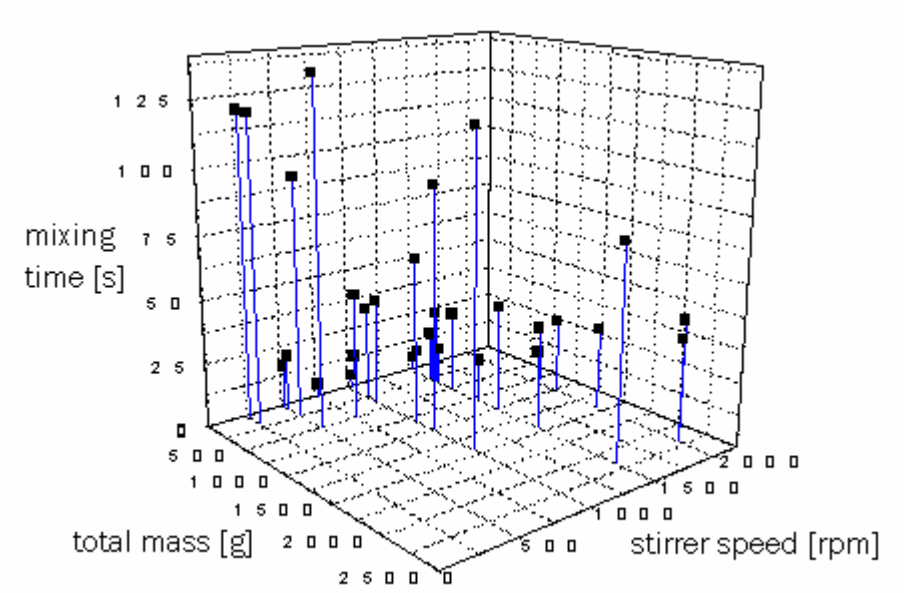


Fig 3.12.1: Mixing time for microemulsion depedent on total volume and stirrer speed

Qualitative experiments with different stirrer types (propeller, disk, Mig, any of these in stacked formation) showed only minor differences apart from different baffle demand. In order to keep the mixing time below the estimated treshold of about 10 seconds, it is necessary at higher volumes to create a situation where large amounts of air are sucked into the body of liquid without at the same time letting the stirrer run dry or creating violent breakers at the surface that could do damage. The latter was a problem especially with stacked 2-bladed stirrers with the topmost blades too near the surface. The best results were achieved with an oversized (17 cm diameter in a 19 cm vessel) three-bladed propeller (flat elliptical blades) running at 250 rpm near the bottom of the reactor with 4 installed baffles that reach down to just above the stirrer level.

A suitable technical solution is therefore to introduce the water phase at a very slow flow (experiments suggest flows <1ml/min), at a distance from immobile surfaces, and at a point of high turbulence/high shear created by a fast running auxiliary stirrer directly below the feed (Fig.3.12.2). That stirrer could be switched off, when no (unemulsified) aqueous compound is currently pumped.

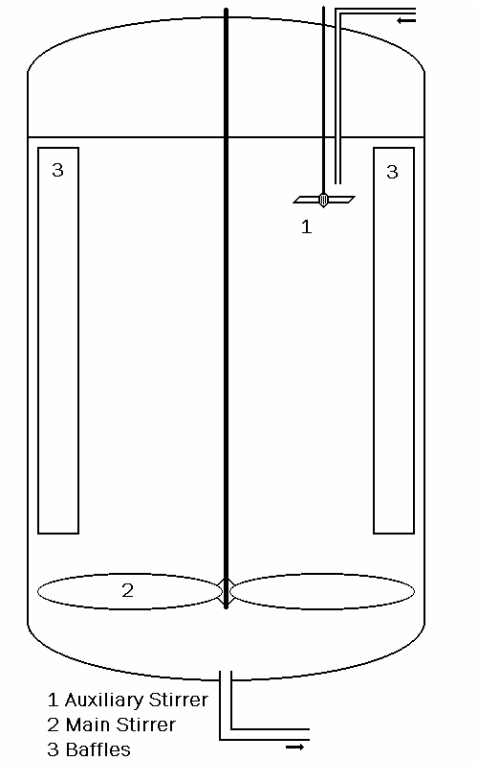


Fig. 3.12.2: Proposed reactor configuration for mixing of microemulsions that minimizes the formation of metastable phases

3.13 Ultrafiltration of w/o Microemulsions

In this study only porous membranes were used. Those consist of a thin "active layer" with defined narrow pores on a support without filter function in the desired particle size range. They are therefore also called asymmetric membranes. Those used here were either made of metal oxides (ceramic membranes) or from organic polymers. The first group was without exception hydrophilic in nature, the latter contained both hydrophilic and hydrophobic membranes. This does not purely depend on the basic material but in some cases on functionalities grafted on the surface of a wide-pored standard model.

The selection was limited by the solvent stability of different membrane materials. It had to withstand the contact with both apolar hydrocarbons (cyclohexane) and water as the basic ingredients of the filtered solutions. Additionally a certain concentration of ketones

had to be tolerated (substrate for the enzyme reaction). The interaction with surfactants, wherefore only limited data is available, had to be tested individually.

The ceramic membranes are the only ones that are completely non-problematic concerning the solvent. Most organic membranes on the other hand are very sensitive to mixtures of polar and apolar compounds. Many materials able to withstand alkanes do either swell or are even dissolved in presence of ketones, especially acetone. While this may be tolerable for the support material to a degree, it is not for the active layer because it leads to either a blocking of the pores (no flow) or to their destruction (no filtration effect). As a result all membranes that use unprotected modified cellulose in the active layer proved unusable.

To a lesser degree the same problem occurs with the elastic seals in both the ultrafiltration cell and the pressure keeping valve. Commercial filtration units also often use materials sensitive to ketones, e.g. the lute in fibre modules. For that reason ketone concentrations have to be kept well below 5 Vol.-%, with short-chain compounds being more critical than e.g. acetophenone.

Ceramic membranes have two main disadvantages. They are fragile and break easily under tension. They are also much thicker than the equivalent polymer membranes and can absorb significant amounts of dissolved components. This can both distort the mass balance in the current experiment and taint subsequent ones. In the case of the DisRAM Inside® (TAMI) ZrO_2 membrane (also the thickest tested) this was demonstrated in an especially conspicious way.

An o/w emulsion of water insoluble PAN (1-(2-Pyridylazo)-2-naphthol) was filtrated in cross-flow mode. For about 20 minutes the retention oft he dye was 100%, i.e. the permeate was completely colorless. Then the retention dropped to zero within less than a minute. The membrane itself showed a strong red coloration that could not be removed by simple rinsing of the surface. The membrane was reinserted into the filtration cell and filtration restarted with a dye-free solution. The permeate was strongly tainted with the slowly desorbing dye. Even after an estimated rinsing time of 100 hours, the dye could still be detected and there remained a residual discoloration on the membrane surface (the latter remaining constant).

The other ceramic membranes with the exception of the Kerafol $MgAl_2O_4$ samples showed a similar "breakthrough" effect in the range of 10-20 minutes and also acted as temporary absorbers, although to a lesser degree. The initial higher retention could theoretically be maintained by switching the filtration between several membranes about every 10 minutes. This is not practical though because the rinsing of a membrane to

reverse the effect takes significantly longer than the build-up. In extremis this would mean one channel for filtration vs. more than 10 for rinsing. Water-soluble dyes were also washed out more easily than PAN. The Kerafol $MgAl_2O_4$ showed no retention from the start on, owed arguably to the rather large pores (7 nm, cut-off 15 kDa) that considerably exceed the typical micelle diameter. This can nonetheless be only a partial answer because the TAMI ZrO_2 membrane has the same nominal cut-off. The difference may be explained by the fact that the Kerafol $MgAl_2O_4$ membrane had only about half the diameter (47 mm vs. 90 mm for the other ceramic membranes) and a lower thickness, thus reducing the potential absorption volume by a factor of 5-6. It is likely that the decsribed behaviour is due to a combination of two effects. The first is the breakthrough of the active layer and is related to interactions of the membrane surface, the used surfactant and dissolved components. This will be described in more detail below. The second is the total and relative absorption capacity of the support material. Polymer membranes are thinner providing a lower volume but may show a higher affinity to organic dyes. They are therefore more likely to permanently absorb a small quantity of dye while the ceramic membranes will store larger amounts but only temporarily.

The remaining ceramic membranes were donated by hitk. Those were produced from TiO_2 (d_{50} = 0.9 nm), ZrO_2 (d_{50} = 3 nm) and SiO_2 (d_{50} = 1 nm) resp. with a uniform diameter of 90 mm and a thickness of 1 mm.

Here the tests were first carried out with a cyclohexane based microemulsion of 10-12 wt-% surfactant loaded with the near maximum of water (w_0 ~ 10) in order to maximize the micelle diameter while still keeping the viscosity at an acceptable level. Later the microemulsion was additionally dyed with Coomassie Brilliant Blue. Malachite Green showed a higher bleaching rate in presence of the ceramic material and was therefore unsuitable. The membranes with 7 nm pores showed a pressure drop equal to or smaller than the pressure keeping valve used to control the pressure in open position (0.7 bar). This required a second valve on the permeate side. Membranes with narrower pores on the other hand required a higher pressure on the retentate side to create a sufficient filtrate flow. In the case of the TiO_2 (0.9 nm) membrane at a pump rate of 20-25 ml/min a pressure of about 8 bar was necessary to create a permeate flow of 2 drops per minute (~ 0.1 ml/min < 1 l/m^2*h). At 3 nm a pressure of 2-4 bar was sufficient to create a flow of 1-2 ml/min (~ 10-20 l/m^2*h).

Ceramic membranes with 7 nm pores showed zero retention for water in the long term, i.e. after the breakthrough period. At 3 nm there was a slight retention at lower pressure (never above 10-15%) dropping to zero once the pressure went above about 3 bar. The

TiO_2 membrane actually showed a negative retention of 10-20%, i.e. the water concentration in the permeate was actually higher than in the fed solution. This was demonstrated, apart from the rather imprecise determination of the water concentration via IR, by the formation of a second phase in the permeate indicating an oversaturation of the microemulsion. A crosscheck showed no significantly lower surfactant concentration, excluding that as a reason. Water seems not to show a distinct breakthrough hiatus with ceramic membranes. The mechanism of passage is therefore probably different from that affecting other dissolved components. It can be assumed that the ceramic material is hydrophilic enough to compete with the surfactant and to withdraw micellarly stabilized water. Thus there would be an increased water concentration on and in the membrane causing the same in the permeate flow. Dissolved dyes accumulate preferably in the surfactant-rich phase. Their behaviour is therefore probably based more on interaction with the surfactant, less with the membrane itself.

This is corroborated by the quite different behaviour towards organic polymer membranes. Those are on average hydrophobic or at least less hydrophilic than the ceramic ones. Water would therefore not accumulate on them naturally but interact through the surfactant only. This seems to be the case indeed because there is no obvious hiatus between the breakthrough of dissolved dyes and water.

The polymer membranes are much thinner and have therefore a far smaller volume for potential absorption. The breakthrough period is measurably shorter as is the time and amount of pure solvent necessary to restore the effect. While it takes many hours to rinse the used ceramic membranes sufficiently to achieve a similar effect as with the fresh one, polymer membranes can be usually reused within about half an hour.

A reasonable explanation is that surfactant molecules from the emulsion adsorb on the membrane surface (a process known as fouling) and thus shield compounds in the solution from polar/apolar interactions with it. On a hydrophobic surface the polar surfactant heads would stick out effectively hydrophilizing it, while on a hydrophilic surface it would be the tails resulting in a partial hydrophobization. In the latter case the actual effect on water would be low though because it could be expected to have an even stronger affinity to the membrane material than the surfactant. But in the former case it would allow hydrophilic compounds to pass through more easily, in essence "leap-frogging" over the polar surfactant heads.

Results for water retention with tested modified organic polymer membranes are displayed in table 3.13.1+2. For comparision information by the provider about flow tests with pure water are given.

Table 3.13.1: Tested organic polymer membranes of PolyAn company (first series):

membrane	modifying monomer	DG(mg/cm²)	H_2O flow (l/hm²) according to provider	measured H_2O retention
PAN unmod		0	283,2	No retention
PAN 031003/2	MePEG200MA	0,15	none detected	13% /5bar*
PAN 031003/4	MePEG200MA	0,11	21,3	?
PAN 031003/6	MePEG200MA	0,11	75,7	31% /2.5bar#
PAN 030702/1	ChxMA	0,26	49,4	17% /1 bar§
PAN 030704/2	MePEG200MA/CHxMA	0,23	175,5	20% /2-3 bar

* otherwise insufficient flow; # permeate < 1% of retentate, i.e. minimal flow; § singular result of 58% at 2.5 bar irreproducible

For the second series reliable values could only be determined for PAN 031401/3 , for /1 no retention was detectable, for /5 the flow proofed insufficient. PAN 031403/1 and /3 seem unsuitable due to very low flows of 10-20 ml/h filtrate compared to a total cell flow of 50 ml / min. For the given membrane size 1ml/h permeate flow equals 0.5 l/hm²

Table 3.13.2: Successfully tested organic polymer membranes of PolyAn company (second series)

membrane	modifying monomer	DG (mg/cm²)	H_2O flow (l/hm²) according to provider	measured H_2O retention
PAN 031401/3	CHxMA	0,67	138,98 moisted with MeOH	10-20%/ 1bar 10-20ml / h

A mixed organic/anorganic membrane (brand name Creavis) was obtained through the same provider (no further information available from original producer Degussa). Test results were of very low reproducibility but even the best retention rates measured were not sufficient to justify further efforts.

As a general result hydrophilic membranes (that includes all ceramic membranes tested) turned out to be completely unsuitable for the filtration of w/o microemulsions, while the case for hydrophobic (organic polymer) membranes seems to be more complex. Pore diameters seem to have to be lower than 3 nm to show any retention effect at all. This is also in agreement with micelle sizes obtained by DLS. Trans-membrane pressure should not to exceed 1 bar. Resulting low flows have to be compensated with large filtration areas. The transmembrane pressure has to be kept low (ideally < 1 bar) or micelles will pass through even with nominally higher diameters than that of the pores.

3.14 PVA gels, characteristics and use for enzyme immobilization

3.14.1 PVA gels as support for the immobilization of enzymes

Membranes made from poly(vinyl alcohol) (PVA) based gels have been discussed as support materials for the immobilization of enzymes earlier [Orlich 2000(1), Djennad 2003]. The usual methods are the production of gels by either a physical process of repeated freezing and thawing of a PVA solution (more commonly a filter paper soaked with the solution) or the same in combination with chemical crosslinking by glutardialdehyde catalyzed by small amounts of hydrochloric acid. Enzymes, esp. lipases, can be immobilized on this support by the same crosslinking process. Membranes produced with this method have a pressure drop low enough to be used for reactive filtration ('reactive membranes'). Since attempts to produce thicker layers (in order to make the filter paper support unnecessary) showed problems with homogeneity and reproducibility, experiments have been conducted in order to determine, whether the freezing-thawing procedure could be avoided and the gel/membrane production carried out at room temperature without significant changes in the properties of the material. In this case only the chemical crosslinking could be used. It was found that the gelation at room temperature mainly depends on two factors that do not play a significant role in the traditional method. Those are the amount of acid catalyst used to initiate the gelation and the removal by evaporation of excess water. While in the freezing-thawing process only tiny amounts of diluted hydrochloric acid are sufficient to produce a stable gel, the gelation at constant (room) temperature requires much more.

Using 5%(w/w) solutions of PVA and 25%(v/v) solutions of glutardialdehyde(GDA), the relative volumes employed for the typical experiment are 40:1:0.5-1 PVA/GDA/fuming HCl. If the amount of acid is reduced below that, gelation is slowed significantly or does not occur at all (not within 2 weeks). If on the other hand the acid is not quickly dispersed in the mixture of PVA/GDA, gelation occurs instantly at the point of entrance (but not beyond that). Acid is therefore added at last to the vigorously stirred mixture. If higher amounts of GDA are used, solidification is achieved within 20 minutes. Under conditions as given above the mixture will grow ever more viscous and stop flowing within a few hours. The produced colourless, clear, jelly-like substance still contains the complete water. Depending on the amount of GDA used and the relative area of surface it will undergo a second transformation. At high GDA concentrations the gel shrinks within a day by at least a third and loses the excess water. The transparency is partially lost and the gel takes a slight blue tone. If kept moist, no further change can be observed (the gel is sensitive to growth of mould under this condition, though).

Fig. 3.14.1.1 Samples of crosslinked PVA gels produced by RT method
left : foil after repeated moisting and drying
center front : foil after drying
center back: block produced at high crosslinker concentrations
right: foil produced at very high crosslinker concentrations

If allowed to go dry, the substance shrinks further slightly and loses its flexibility completely. It also turns to brownish yellow (Fig.3.14.1.1 center back) or even (at extreme excess of crosslinker) black (Fig.3.14.1.1 right). This process cannot be reversed. Experiments with enzyme immobilization have been carried out with the moist shrunk stage (see below). At lower GDA concentrations as given above, the shrinking process does not occur while the gel is kept moist. If allowed to dry, a shrinkage of 40-60% can be observed. The transport of the excess water inside the gel is very slow. A block thicker than about 5 mm will dry only at the exposed surface by slow evaporation of water.

Experiments with dye solutions show that water from the outside can't penetrate the moist gel even under pressure, although dye can bind to the gel permanently (Fig.3.14.1.2 center left). Aqueous Coomassie Brillant Blue that has been brought into contact with dry or moist gel can't be washed off by either water or organic solvents (alcohols, ketones, cyclohexane).

Thin layers (<~2.5 mm) cast on a smooth surface (glass, metal, plastic) will dry within 2-3 days and shrink vertically almost exclusively, thicker layers will either 'roll up' at the sides or crack (on not completely planar surfaces, circular holes can appear). The dried gel (clear, colourless) sticks to the surface but can be pulled off without damage. It is stiff, inelastic but flexible and very resistant to tear (Fig.3.14.1.1 center front). Acceleration of drying by increased temperature (60°C) leads to a dark brown colouration and a less smooth surface but does not change the other characteristics (Fig.3.14.1.2 sample A).

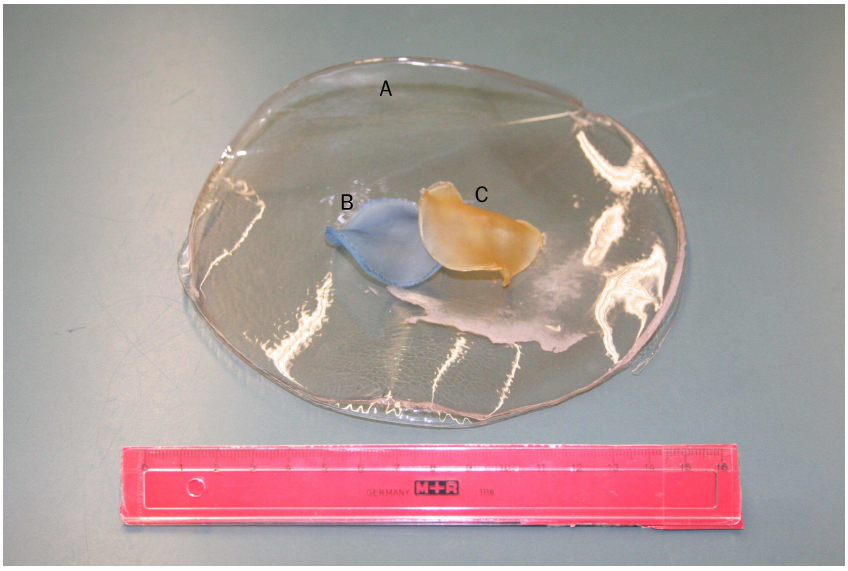


Fig. 3.14.1.2 Samples of crosslinked PVA gels produced by RT method
large sample (A): foil dryed at 60°C (brown color through slight decomposition)
small samples:
center left (B) : thick foil dyed with Coomassie Brilliant Blue during gelation
center right (C): thick foil produced at moderately high crosslinker concentrations

If brought into contact with water, the foils expand (mainly laterally) and loose both their stiffness and tearing resistance. Water will not pass through the foil in liquid form, though, even under pressure (18 bar, safety limit of the ultrafiltration cell), as experiments in ultrafiltration cells have shown. The process is reversible (although the uneven shrinking/expanding may change the geometry to a certain degree as can be seen in Fig.3.14.1.1 left).

3.14.2 Surface structure of crosslinked PVA gels

Samples of bulk and carrier supported crosslinked PVA gel (cPVA) were analyzed by Scanning Electron Microscopy (SEM). Those on carrier were additionally freeze-dried before SEM inspection, the others only exposed to dry air for a longer time (several weeks).

The depicted samples can be classified as follows:

1. Foil prepared at room temperature with immobilized lipase (Fig.3.14.2.2+4)
2. Foil prepared at room temperature without immobilized lipase (Fig.3.14.2.3)
3. Lump contracted due to high amount of crosslinker (Fig. 3.14.2.13)
4. Filter paper coated/drenched with cPVA at room temperature (Fig.3.14.2.1+6+11+12)
5. Filter paper coated/drenched with cPVA using the freezing-thawing method (Fig.3.14.2.5+7+8+9+10)

Samples with and without immobilized enzyme were undistinguishable, i.e. enzyme complexes were not visually detectable on the surface.

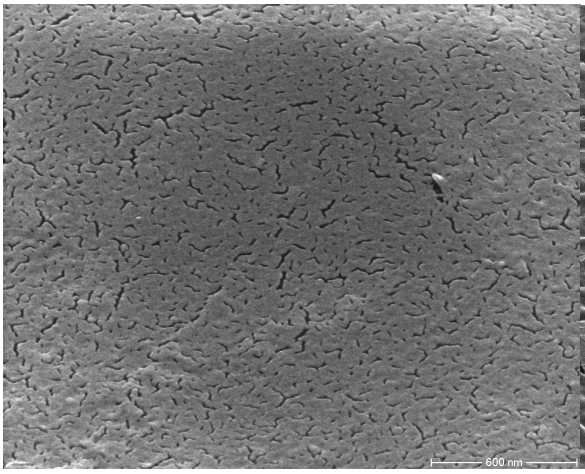


Fig 3.14.2.1, Magnification 50000x,
cPVA on filter paper support
crosslinked and dried at room temperature,
freeze-dried before SEM inspection

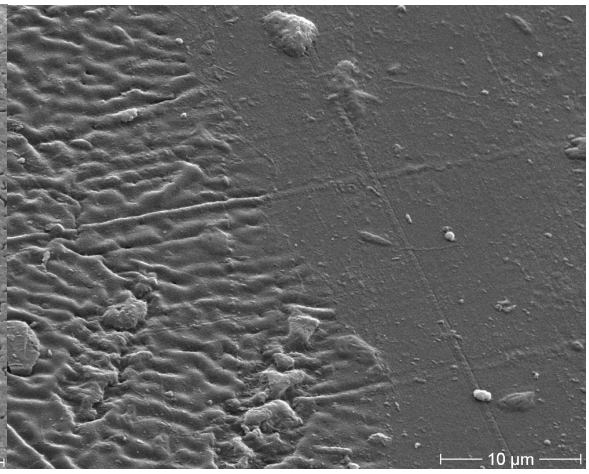


Fig 3.14.2.2, Magnification 3000x,
cPVA cast as bulk on plastic surface
crosslinked and dried at room temperature,
not freeze-dried

All samples have in common that at the highest possible resolution a pattern of cracks is visible (cf. Fig 3.14.2.1). These are structurally similar to those created when the electron ray is focused on an area of the sample other than briefly. It is therefore concluded that those are a result of the drying process and will close when the material becomes humid. This unfortunately cannot be tested because the SEM devices available require dry samples.

The main surface features of the bulk cPVA can be attributed to the surfaces the samples were cast on (Fig.3.14.2.2), otherwise they are almost perfectly smooth and featureless (Fig.3.14.2.3). A cut through the foil shows that the material is dense and has no macropores (Fig.3.14.2.4). This agrees with the observed impermeability for liquids. The "pine tree" patterns are probably a result of the asymmetrical strain the material underwent during the drying process (free contraction in height, inhibited horizontal contraction due to adhesion to cast surface). Strain marks but without much lower anisotropy are visible also in the sample where the surface adhesion was overcome by the contraction as a result of significantly higher amounts of crosslinker used (Fig.3.14.2.13).

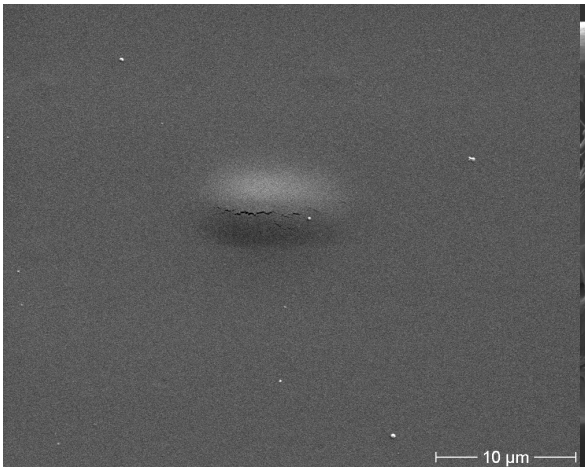


Fig 3.14.2.3: Magnification 3000x,
Bulk cPVA foil
crosslinked and dried at room temperature,
not freeze-dried
Center crack caused by electron ray of SEM

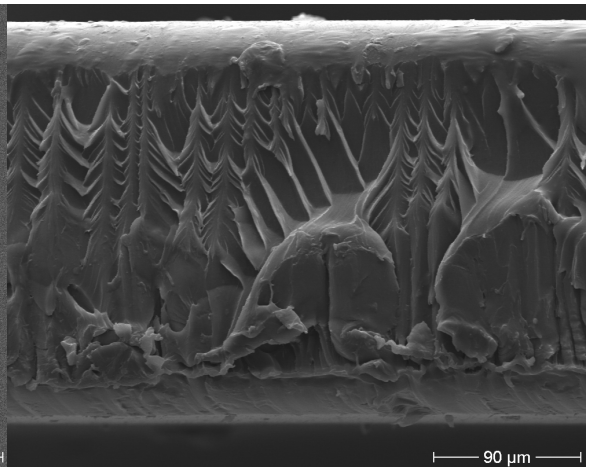


Fig 3.14.2.4: Magnification 350x,
Cut through bulk cPVA foil
crosslinked and dried at room temperature,
not freeze-dried

The samples of cPVA on a filter paper carrier show distinctly different features except at the highest resolution (cf.above). Some of these are possibly artefacts caused by the freeze-drying and not to be found under normal circumstances (cf. below).

In all cases a part of the cPVA forms a thin surface layer, the rest has penetrated the support and coats the cellulose fibers *without* filling the space in-between (Fig.3.14.2.5+6). This surface coat is therefore to be considered the main resistance factor to flow of liquids through the membrane.

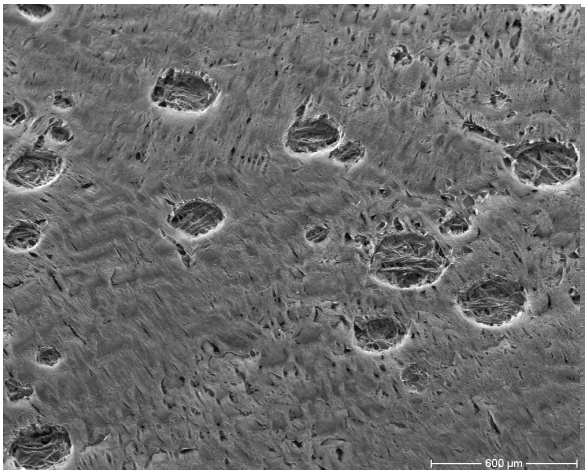


Fig 3.14.2.5: Magnification 500x,
cPVA on filter paper support
crosslinked & dried by freezing-thawing meth.
freeze-dried before SEM inspection

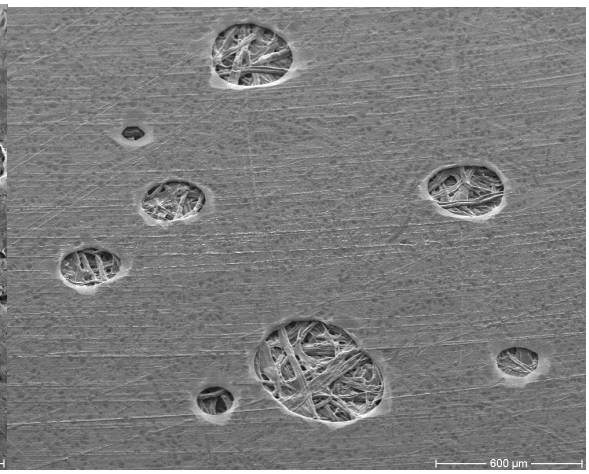


Fig 3.14.2.6: Magnification 500x,
cPVA foil on filter paper support
crosslinked and dried at room temperature
freeze-dried before SEM inspection

Room temperature and freezing-thawing samples show clear differences that concur with their membrane characteristics. Samples prepared by the same method on the other hand are very similar, i.e. the particular features are reproducible.

Room temperature samples have a smooth and mostly unbroken surface of varying thickness (signaled by changes in the albedo). The "crater" (Fig.3.14.2.6) structures are very rare and are probably the result of bursting surface bubbles caused by the applied vacuum during the freeze-drying process (they are absent in samples not undergoing that procedure).

The freezing-thawing samples on the other hand show a very rough and pockmarked surface (Fig.3.14.2.7). The craters are far more numerous and there are also cylindrical holes to be found penetrating the surface coating completely (Fig.3.14.2.8).

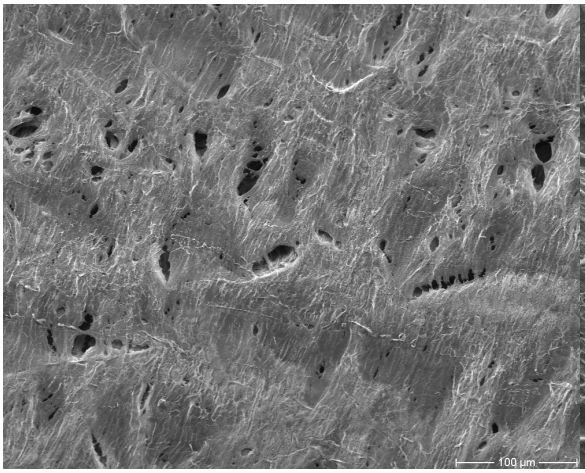


Fig. 3.14.2.7: Magnification 500x,
cPVA on filter paper support
crosslinked & dried by freezing-thawing meth.
freeze-dried before SEM inspection

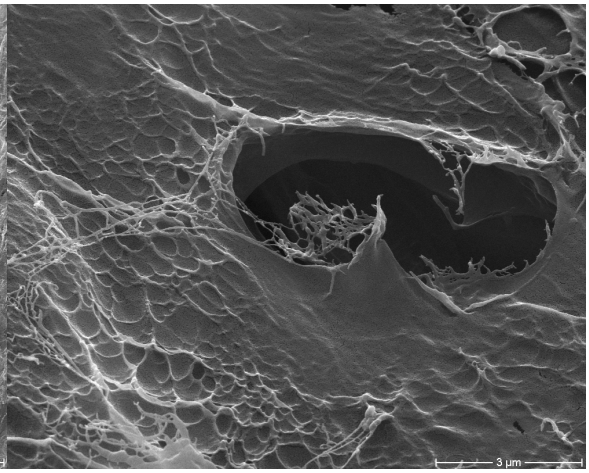


Fig.3.14.2.8: Magnification 10000x,
cPVA on filter paper support
crosslinked and dried by freezing.-thawing method
freeze-dried before SEM inspection

There is currently no explanation for the net-like structures (Fig.3.14.2.9+10) that seem not to be part of the surface in general but cover large areas.

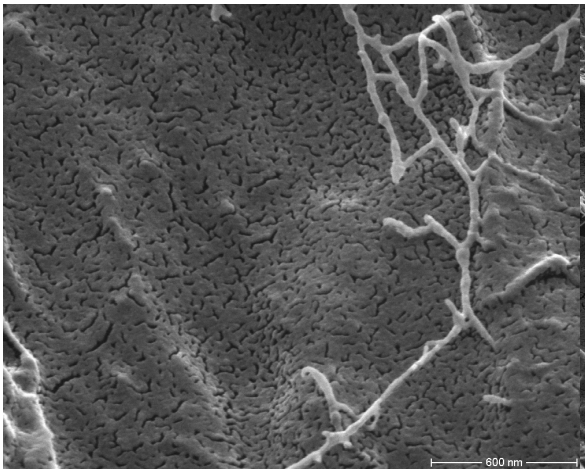


Fig.3.14.2.9: Magnification 50000x,
cPVA on filter paper support
crosslinked & dried by freezing-thawing meth.
freeze-dried before SEM inspection

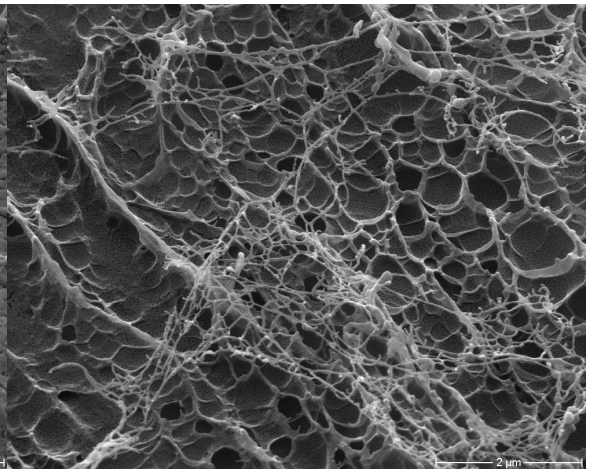


Fig.3.14.2.10: Magnification 15000x,
cPVA on filter paper support
crosslinked and dried by freezing-thawing method
freeze-dried before SEM inspection

3.14.3 Conclusions from the SEM analysis

Samples of cPVA produced at room temperature have a dense structure lacking macropores. They are therefore impervious to liquid water although they can take up a certain amount by swelling provided the amount of crosslinker did not exceed a maximum level (not determined in this study). On filter paper support the cPVA forms only a very thin surface coating (nm range). Inside the support there seems to be only a coating of fibers (Fig.3.14.2.11+12), a surface treatment should therefore make the membrane permeable.

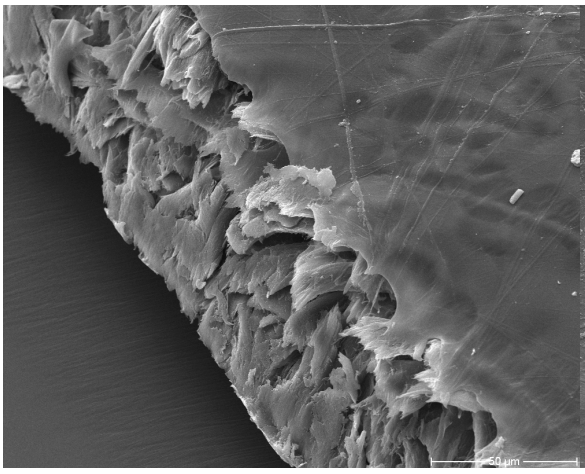


Fig.3.14.3.1, Magnification 600x,
Cut through cPVA on filter paper support,
crosslinked and dried at room temperature
freeze-dried before SEM inspection

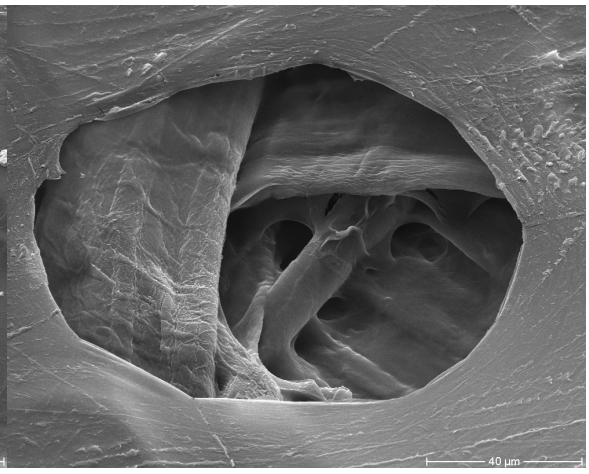


Fig.3.14.3.2, Magnification 750x,
cPVA on filter paper support
crosslinked and dried at room temperature
freeze-dried before SEM inspection

Bulk cPVA is not suitable for membranes but may be used for the thin coating of egg-shell catalysts. Samples produced by the freezing-thawing method (only on support) also form

a surface coat but this is highly fractured in nature thus allowing the permeation of liquid water. The extreme differences in permeability are therefore easily deduced from the visible microscopic structures. Although enzymes can be and were immobilized successfully on the cPVA, they were not visible in the SEM pictures.

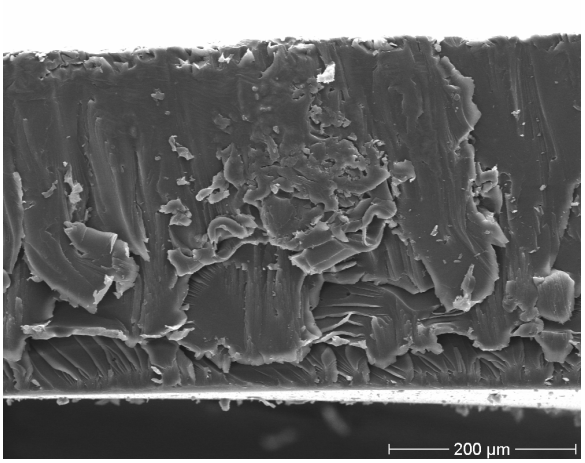


Fig.3.14.3.3: Magnification 200x,
highly contracted cPVA as result of high crosslinker concentration, prepared and dried at room temperature, not freeze-dried

3.14.4 Immobilization of lipase on crosslinked PVA gel

Lipases can be immobilized on PVA gels using the same agent (glutardialdehyde) as used in crosslinking the gels for stabilization [Tischer 1999; Chae 2000; Chauhan 2004]. For ADH enzymes the method as used here seems unapplicable due to the far from neutral pH conditions necessary. While lipases can withstand the low pH values for the time needed for immobilization, the available ADH enzymes cannot, i.e. their activity is lost completely. All experiments described were therefore carried out with *Candida rugosa* lipase (CRL). Although the use of GDA is very common for enzyme immobilization due to its easy applicability, it has been criticized. Chase[1998] observed a higher loss of activity compared to other methods. El-Zahab[2004] found a connection between the activity of immobilized oxidoreductases and the length of the spacer used. Short spacers (as GDA) yielded the lowest results.

Djennad et.al.[2003] used PVA gels produced by the freezing-thawing process (as described by [Muscat 1996] and [Lozinsky 1998]) and fixed on filter paper as support. These could then be tested as reactive membranes in filtration cells. The CRL was immobilized on the membranes after their production not during the gel formation. As described in chapter 3.14.1 the freezing-thawing process was abandoned and the gel formation carried out at room temperature.

In the first series of experiments the gel was produced in bloc form in crystallizing dishes

and then cut to the intended size. Adding the enzyme during the gelation process resulted in complete loss of activity. This loss is not caused by denaturation due to the low pH values as control experiments show. The probable reason is two-fold. On the one hand the enzyme is entrapped in the gel and, as other experiments with PVA gels have shown (chapter 3.14.5), cannot be reached by the substrates (dense gel). On the other hand the enzyme could be distorted in its structure by the excess of crosslinking agent present, i.e. multi-point anchoring preventing the enzyme undergoing the induced-fit step necessary for its operation. All further experiments therefore separated the gel formation and the enzyme immobilization steps.

Moist gel was cut to cubes of 2-3 mm and put into a stirred tank reactor. Enzyme solution, glutardialdehyde and hydrochloric acid (catalyst) were added and the mixture stirred for 20-30 minutes. Afterwards the liquid phase was gradually replaced by pure water until no enzyme activity could be detected in the aqueous solution anymore and pH neutrality was reached.

In order to test successful immobilization a micellar solution containing para-nitrophenyllaurate (pNPL) was fed to the stirred reactor and the outgoing stream tested for paranitrophenol. The results were negative independent of residence time.

Next attempts were made to coat glass beads (2-3 mm) with a thin layer of PVA gel by simply immersing them in the gelating mixture and allowing it to dry (Fig.3.14.4.1).

Fig.3.14.4.1: Samples of crosslinked PVA gels (RT method) on glass beads
left: separated coated beads for fixed bed experiments
right: glass beads embedded in crosslinked PVA gel

This was only partially successful, the coating was uneven and often did not cover the complete bead. The coated beads were filled into a glass column and the standard

mixture of enzyme, crosslinker and acid was slowly pumped through for about 20 min. The swelling of the gel on contact with water significantly increased the pressure drop and caused an uneven flow through the bed. After cleansing with pure water the pNPL test solution was pumped through with a residence time of a few minutes. A change from colourless to yellow clearly indicated the formation of nitrophenol, i.e. observable enzyme activity. Control experiments with coated beads without immobilized enzyme showed no reaction. Kinetic measurements were not taken because of the above mentioned imperfections of coating and regular flow.

These experiments show that it is generally possible to use the enzyme in a PVA gel based shell catalyst fixed bed reactor but the technical feasability is doubtful. All further experiments concentrated on the immobbilization of the CRL on thin PVA foils/membranes.

The solid PVA gels produced at room temperature proved dense independent of the amount of crosslinker or the PVA concentration used. Therefore dried gel foil was cut to ribbons and put between two layers of filter paper (perforation would serve the same purpose). The enzyme immobilization was then carried out the same way as with the membranes produced by the freezin-thawing process. These "membranes" were tested in the ultrafiltration cell in the continuous (dead-end) mode as decribed by Djennad [2003,2005]. The observed mass-related enzyme activity was comparable to that yielded by the conventional method.

The quantitative results of both this and the older study are partially compromised by the leaching effect. Despite rinsing of the fresh membranes with first BSA and then pure water in order to remove enzyme not chemically bound to the gel prior to testing there is measurable enzyme activity in the stream leaving the filtration cell. That means that there is further reaction during the time necessary to collect enough sample for quantitative analysis. Additionally there is a (though very low) rate of reaction even in apparent absence of enzyme carrier in the cell. This could be caused by contamination that is resistant to rinsing efforts.

This demonstrates again the notorious problem of enzyme immobilization on carriers. Leach-proof fixation almost inevitably has to be bought at the cost of significantly reduced activity (through distortion of the enzyme geometry) or, if this is to be avoided, a constant loss of enzyme (apart from natural deactivation) has to be accepted.

3.14.5 Other use of crosslinked PVA with immobilized enzyme

Crosslinked PVA produced at room temperature can be used in other forms than membranes. Experiments were carried out to immobilize lipase on or in blocks of gel for use in slurry reactors. The coating of packings for the use in columns has been studied qualitatively.

As described in chapter 3.14.1 water and other solvents (cyclohexane, isopropanol, acetone) are unable to penetrate far into the crosslinked PVA gel, and the internal diffusion seems to be very slow. Therefore entrapped enzyme does not show measurable activity independent of the actual status. It is likely though that entrapped enzyme is deactivated for good due to the presence of residual hydrochloric acid and the high concentration of free GDA during the formation process. All further experiments of enzyme immobilization were therefore carried out after the soldification of the gel and exchange of the surrounding liquid phase. Even after repeated rinsing wet gel samples still indicate the presence of acid by distinct smell and the reaction of pH indicator paper when brought in contact. A neutralization of the acid by a buffer is difficult due to the low mass transport in the gel and would require repeated wetting and complete drying. Immobilization of lipase at the surface of bulk samples was attempted by the method also used for membranes (see above). Contact of these gel particles had no effect on solutions of pNPL. That means that either the enzyme did not become immobilized on the surface at all or that it was not active (or instantly deactivated resp.). This seemed to be in contradiction to the successful immobilization of lipase on membranes or foils produced by the same method.

The crucial difference is probably the fact that the membranes or foils were allowed to dry completely before enzyme immobilization was attempted, while the bulk gel was treated while still moist. In order to test that hypothesis the glass bead coating experiments described above were carried out. Slow feeding of substrate solution to the column led to the typical yellow colour indicating the presence of active enzyme while control experiments showed no activity. This corroborates the hypothesis that the drying process is the key difference indeed. An explanation is still to be found though.

A disadvantage of the column with coated beads is the swelling of the gel coating. It creates a high flow resistance, and the feeding under high pressure has a scrubbing effect because the wetted gel does not stick as good to surfaces as the dry gel.

An alternative would be to work without the support material and to cast packings from the PVA gel itself using higher concentrations of GDA. Under the right conditions a drying gel disc takes on a saddle form spontaneously during shrinking (Fig.3.14.1.2, small

samples). Dyeing experiments show that the surface of these dried samples is suitable for immobilization purposes. The samples also show high durability and are not affected by standard solvents.

A method that is already in commercial use (LentiKats®) is to not actually crosslink the PVA hydrogel but to prevent its dissolution or abrasion by addition of a surface protecting stabilizer. Here the biocatalysts are not immobilized on the surface but entrapped inside the gel. At particle sizes of a few millimeters there is sufficient mass transport possible for standard substrates to reach the biocatalyst but not enough for the latter to be leached (cf. [Capan 2002] or [Durieux 2002]).

3.14.6 Activity of lipase immobilized on solid support

The immobilization of the lipase at the surface of a porous membrane has the advantage to allow short contact times. Formed product can thus be quickly removed from the site reducing possible product inhibition of the enzyme. This also avoids establishment of a possibly unfavorable equilibrium. On the other hand the conversion is likely to be reduced at higher flows, i.e. shorter contact times, because substrate molecules might not come in contact with immobilized enzyme in the short time available. Other variables to consider are the enzyme loading (cf. chapter 2.2.14), the substrate concentration and the density/porosity of the membrane. Narrow pores limit the possible flux but increase the contact probability. Higher enzyme loading will also increase this probability but might lead to negative interaction between enzyme units. It would also allow multiple contacts leading again towards the unfavorable equilibrium state. Since the number of enzyme units defines the maximum total reaction rate (bottleneck through saturation) an increase in substrate concentration is not useful beyond a certain limit. Any increase beyond that limit will thus automatically reduce the conversion. In Fig.3.14.6.1+2 the dependency of the conversion on flux and substrate concentration for two different loadings of enzyme can be seen.

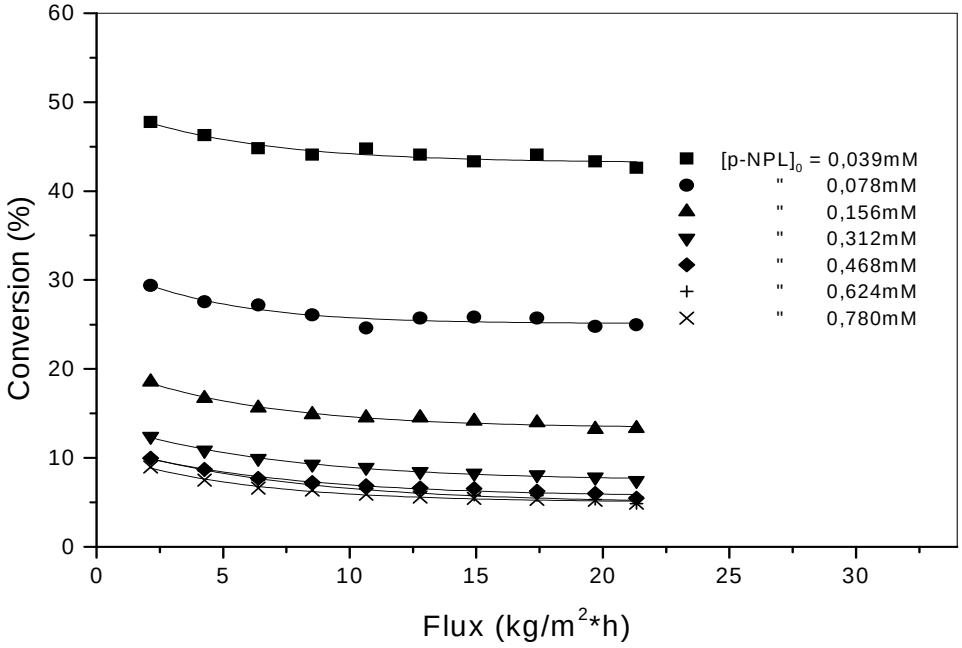


Fig.3.14.6.1: The variation of the p-NPL hydrolysis conversion with the flux and different initial concentrations of p-NPL; loading of enzyme: 2,26 mg/l; membrane made from 5 wt% solution of PVA

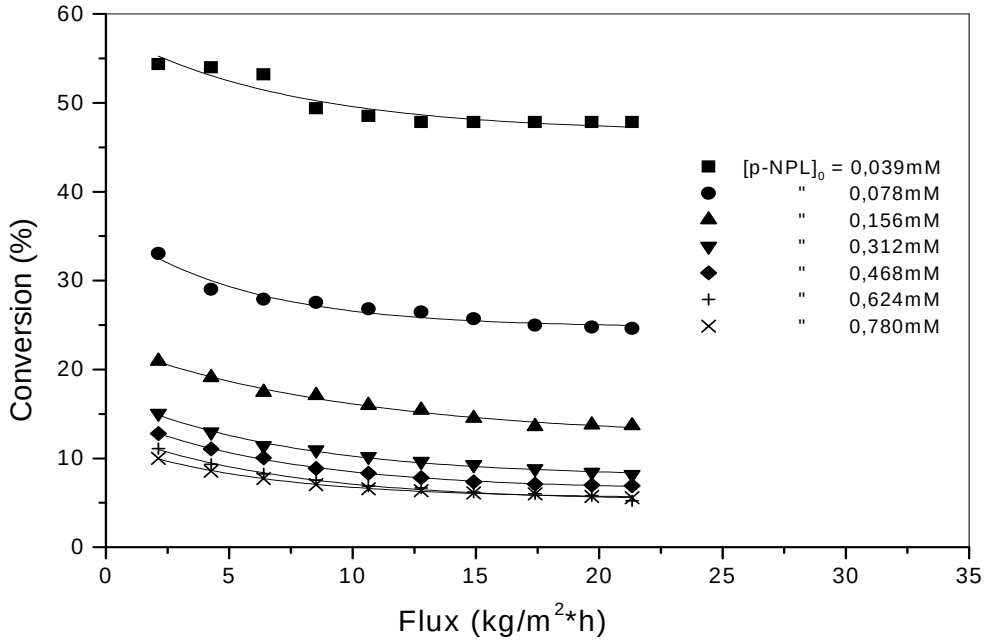


Fig.3.14.6.2: The variation of the p-NPL hydrolysis conversion with the flux and different initial concentrations of p-NPL; loading of enzyme: 4,53 mg/l; membrane made from 5 wt.-% solution of PVA

The conversion decreases with increasing flux and approaches a limit dependent on the substrate concentration. E.g. for the high concentrations the conversion is about halved when the flux is increased by one order of magnitude (conversion 10% → 5% with flux 2.1 → 21 kg/m²h for c(p-NPL)=0.624 mmol/l). The saturation effect can be demonstrated by the roughly fourfold decrease of conversion, when the substrate concentration is increased by one order of magnitude (conversion 25% → 6% for 0.078 → 0.78 mmol/l). The poor solubility of p-NPL in the reaction medium prevents a significant concentration increase in any case.

In order to study the effect of the enzyme loading in more detail, it was varied between 2.6 and 18.12 mg/l as displayed in Fig.3.14.6.3+4. As can be seen, the conversion increases with enzyme loading at first but then goes through a maximum at about 9 mg/l. At even higher loading the conversion decreases again, likely due to unfavorable interaction of enzyme units as known from homogeneous solutions.

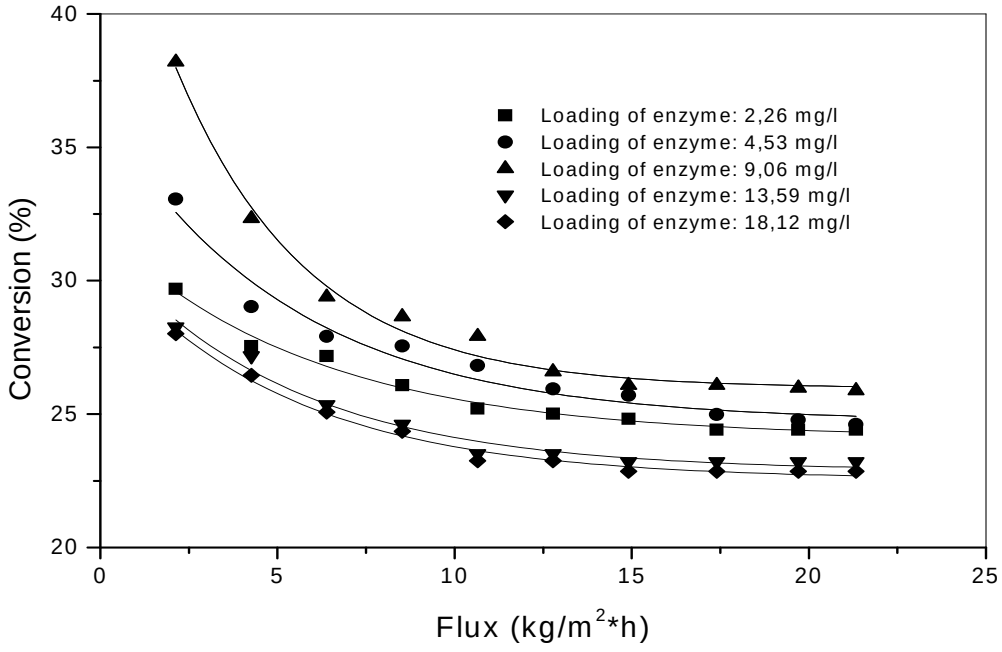


Fig.3.14.6.3: The effect of increased lipase concentrations on p-NPL hydrolysis conversion dependent on the flux for different loadings of enzyme:Cs_0 = 0,078 mmol/l; membrane made from 5 wt.-% solution of PVA

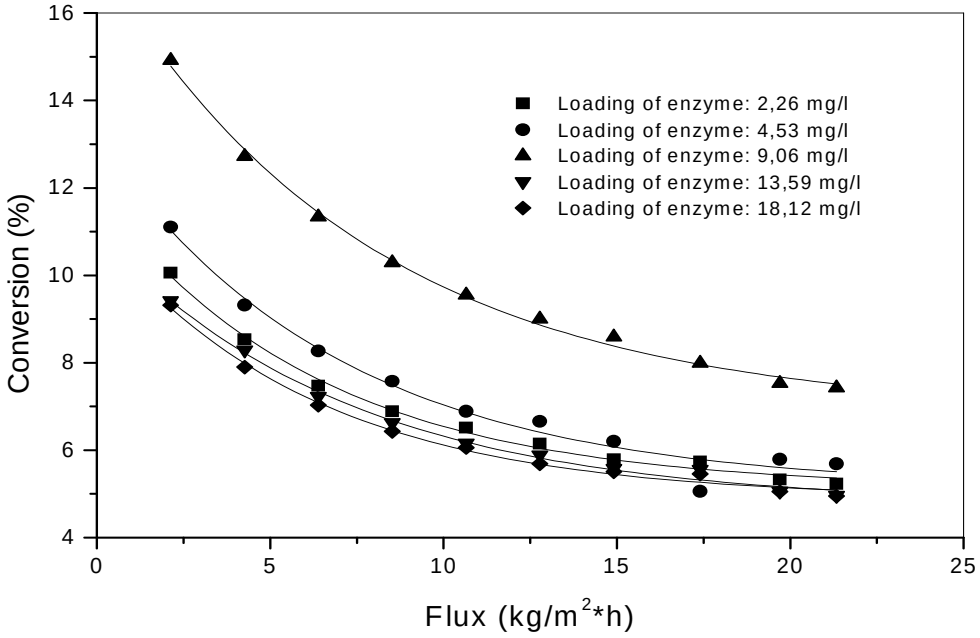


Fig.3.14.6.4: The effect of increased lipase concentrations on p-NPL hydrolysis conversion dependent on the flux for different loadings of enzyme: Cs_0 = 0,624 mmol/l; membrane made from 5 wt.-% solution of PVA

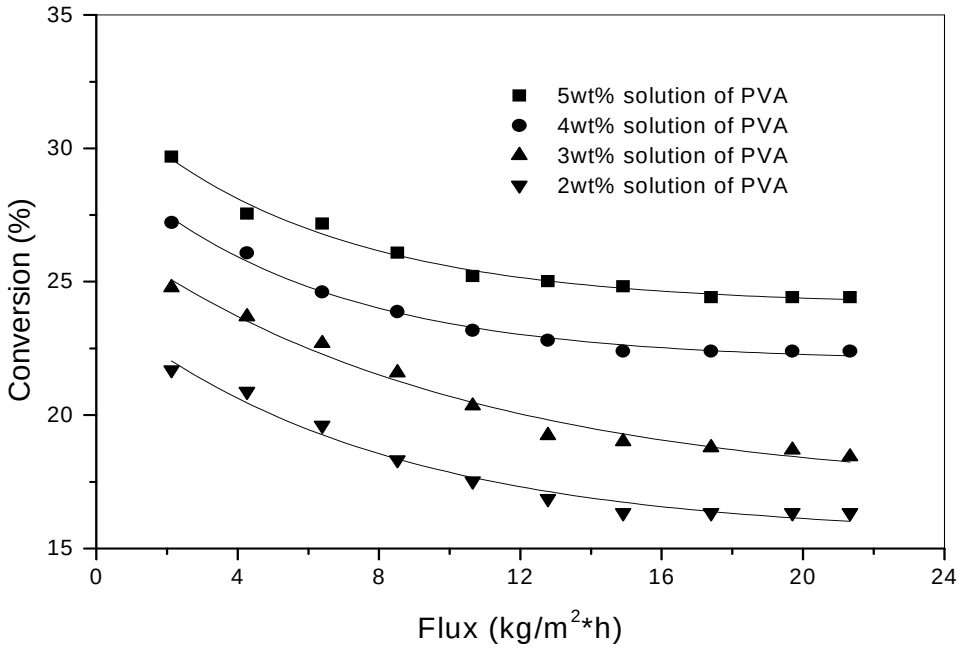


Fig.3.14.6.5: The variation of the p-NPL hydrolysis conversion with the flux for different concentrations of PVA in casting solution at Cs_0 = 0,078 mmol/l; loading of enzyme: 2,26 mg/l

The effect of the carrier material was studied by varying the concentration of the PVA in the casting solution. The higher the concentration, the denser the resulting gels tend to be. This of course under the assumption of equivalent amounts of crosslinker being used. The effect of crosslinker concentration on the properties of the material has been discussed extensively above. As Fig.3.14.6.5+6 show, the conversion increases with the PVA concentration, that is with decreased porosity. This indicates that the assumption made above about narrower pores leading to an increased contact probability has merit. It can also be seen that the effect of the flux is much higher at higher substrate concentrations.

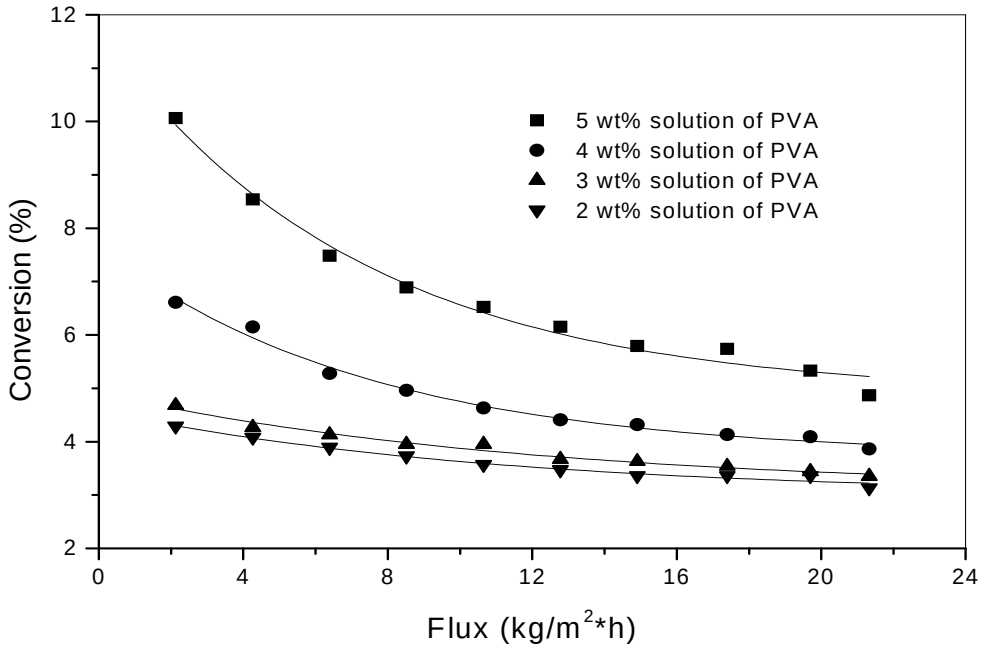


Fig.3.14.6.6: The variation of the p-NPL hydrolysis conversion with the flux for different concentrations of PVA in casting solution at Cs_0 = 0,624 mmol/l; loading of enzyme: 2,26 mg/l

The results of kinetic investigations can be seen in Fig.3.14.6.7. At a flux of 10.6 kg/m²h the maximum reaction rate for immobilized lipase (11.5 U/mg) was found to be comparable to that of free lipase (10.9 U/mg) for the substrate p-NPL under optimum condition (pH8 and 25°C) but the Michaelis-Menten constant K_M is significantly lower (0.069 vs. 0.562 mmol/l). That means that the enzyme-substrate affinity is higher by a factor of 6 for the immobilized enzyme. While the activity of immobilized enzymes is often reduced through forced conformational changes and transport limitations [Tischer 1999], an activation of immobilized lipases is not unknown (e.g. [Goto 2005] reports an 51-fold activity increase in a gel matrix).

It has to be noticed that the kinetic parameters are also dependent on the flux as can be seen in Fig.3.14.6.8. The maximum reaction rate increases significantly with the flux while the K_M value decreases (4.5 U/mg, 0.218 mmol/l at 2.1 kg/m²h vs. 16.5U/mg, 0.046 mmol/l at 21.3 kg/m²h). This indicates other factors to be at work here than just a conformational change of the immobilized compared to the free enzyme. Since at increased flux the effective local concentration of the reaction products decreases (one important reason to adapt the concept of reactive membranes in the first place), one of these factors could be an inhibition of the enzyme by one of the reaction products. This hypothesis has not been tested though.

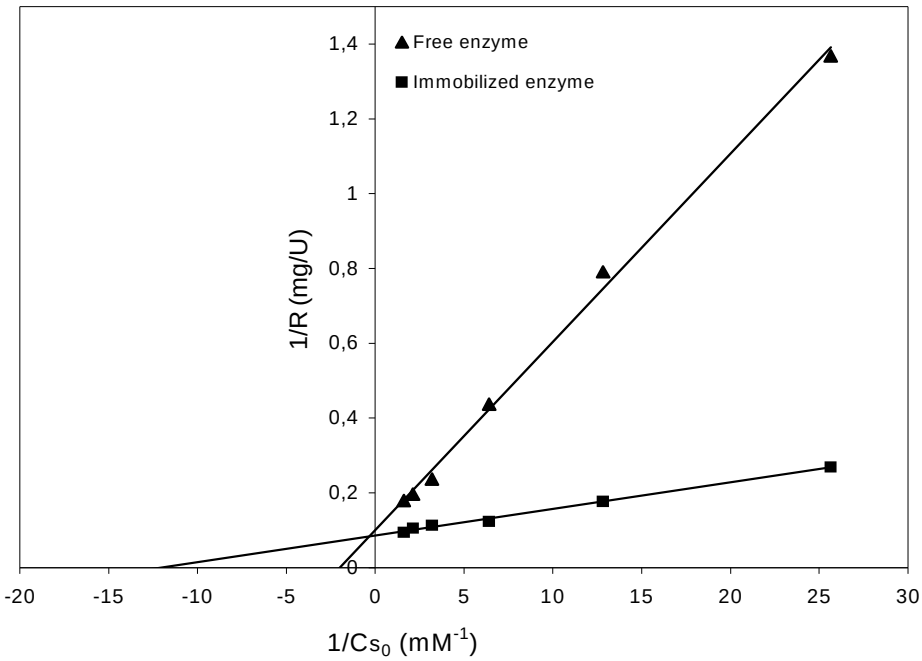


Fig.3.14.6.7: Lineweaver-Burk plots for free and immobilized enzyme to show the effect of the initial substrate concentration on the reaction rate of p-NPL: free enzyme in solution at 3,33 mg/l and immobilized enzyme at 4,53 mg/l loading on membrane made from 5.-wt% solution of PVA and flow rate 10.6 kg/m²*h

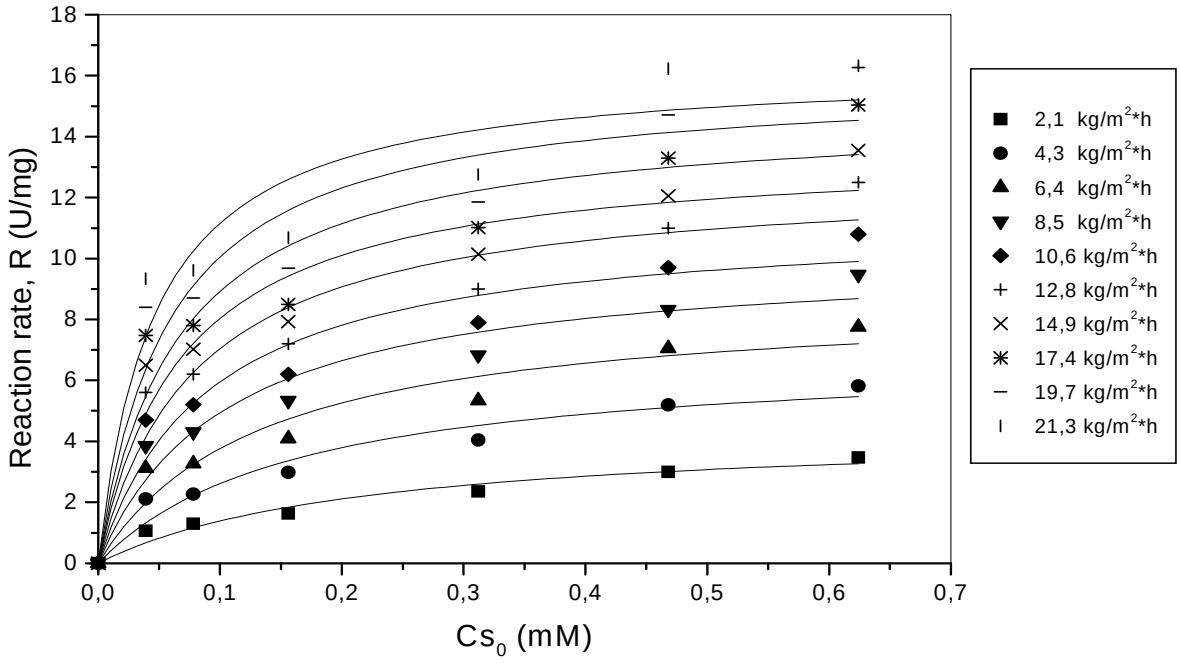


Fig.3.14.6.8: Reaction rate of immobilized enzyme dependent on the flux; membrane from 5 wt% solution of PVA at 4.53 mg/l loading of enzyme

Material from this chapter has been previously published in [Djennad et al. 2003].

IV Conclusion

4.1 The microemulsion route

From the current state of our research it has to be concluded that the microemulsion route is at least not economically viable, even if the practical hurdles could be overcome. Immobilization on a carrier on the other hand may be the way to go.

What now were the main obstacles that made the initially promising microemulsion route an effective dead end?

1. Membranes and Filtration

Given the nature of the reaction mixture, containing polar and apolar compounds, many commercially membranes can't be used because the material does either not withstand chemically or swells uncontrollably. This can be reduced by lowering the reactant (ketone) concentration but it will reduce the longevity, adding costs. Materials that withstand the mixture are unfortunately rarely available with the necessary pore diameter range (there are for example no UF membranes made from Teflon) or they are otherwise unsuitable for the filtration problem. All tested ceramic (=polar) membranes showed low water retention despite sufficiently low pore radii while stable organic polymer (usually apolar) membranes had too wide a pore size distribution. Too narrow pores on the other hand significantly reduce the flow at the low pressures necessary for the integrity of the micelles that otherwise are deformed and thus pushed through. The presence of surfactant also has the effect of a partial reversal of the polar/apolar characteristics of the membranes (stronger on ceramic, lesser on organic). Water thus may pass through the pores of apolar membranes by 'leapfrogging' over surfactant molecules that shield them from the water-repellant walls (for this and similar effects cf. [Krishna 1989, Wesselingh 2000]). The retention of water-bound compounds that are even more critical was found to be on average even worse. Experiments with different dyes showed a qualitative relation between their affinity towards the surfactant (accumulation in the surfactant-rich phase) and the ease with wich they passed through the membranes, corroborating the proposed model. Although a regular rinsing of the filtration membranes could theoretically solve that problem, it would not be practical because the rinsing takes significantly longer (10:1 or higher) than the accumulation of critical amounts of surfactant.

The necessary retention (as per simulation) of 98% of the aqueous phase could not be achieved. Measured micelle diameters require pore sizes bordering on nanofiltration (1 nm or lower) leading to a very low flow in turn requiring very high membrane areas.

Hollow fibre modules that could address that problem suffer from the problem of the lute being sensitive to the organic solvent mixture even if the fibres themselves are not.

2. The surfactants

One major appeal of the approach taken was the assumption that the costs would mainly depend on the enzymes while the chemical environment would consist of rather cheap bulk compounds, technical grade nonionic surfactants in particular. Apart from the price the perceived availability and relative ecological harmlessness were in favor.

As it turned out these assumptions were flawed in several ways. The actual composition of the surfactants changed significantly during the course of this research, unfortunately for the worse, despite the brand retaining its label. On the one hand this consisted of a change in the branching of the oxo-alcohol tail of the surfactant itself, on the other hand an unknown change in the post-synthesis treatment of the raw surfactant increasing the amount and the harmfulness of additives. The highly branched tridecanol of the surfactant batch originally used allowed for a far higher water solubilization of the microemulsion than the moderately or even un-branched available later. The reduction in water capacity (=smaller micelles) negatively affected both the enzyme performance and the filterability of the microemulsion (as described above). The changed additives proved more difficult to remove while having a worse effect on the formation of a clear single-phase microemulsion and probably also reducing the longevity of the enzymes. The additive problem was reduced by a change of the provider but the surfactants available there were from natural resources, i.e. with unbranched alkyl tail. The maximum water capacity (expressed through the $w_{0,max}$ value) was thus reduced by about 50% in comparision to the, now unavailable, original formulation. The purification process proved to be time-consuming and cumbersome while not even achieving a complete success. As a result the promising early experiments could not be reproduced, especially concerning the vital longevity of the ADH enzymes.

3. The problem of upscaling

Two of the studied enzymes were stable enough to perform kinetic measurements in a microemulsion on the cuvette level. The same enzymes worked properly in a batch process with cofactor regeneration in water on the level of a small (0.25 l) lab reactor. Attempts to perform the process on the same scale in a microemulsion failed either completely or the measured activity was much lower than under seemingly identical condition in the cuvette. The lower activity can be attributed to an instant deactivation during the mixing process as experiments have shown. A significant part of the added enzyme is absorbed in the metastable aggregates occuring during the formation of the

microemulsion. In this environment the deactivation is very quick (typically >50% in the first 30 seconds). While in the cuvette the mixing takes just a few seconds, the time for the larger scale is much more extended and also depending on the type and configuration of the stirrer used. The ADH has therefore to be added last to the already formed microemulsion under strong shear forces in order to minimize the contact with the metastable phases. The latter also tend to stick to surfaces and are very resistant to removal thereby complicating even the initial microemulsion formation. This problem can probably be overcome by the combination of a slow stirrer of large diameter installed near the bottom of the reactor and a small high speed stirrer directly at the feed position that is only switched on during the addition of aqueous phase. Thus a sufficiently short mixing time may be achieved without exposing the sensitive enzyme to unneccessary shear stress.

4.2 Immobilization on crosslinked gel carrier

Unlike the problems of the microemulsion route that will probably not be overcome economically, those observed with carrier immobilization are likely to be only temporary. The experiments carried out in this work were in any case of a more exploratory character and less intended to deliver a technically and economically viable process on the spot. But they may serve as a basis for further work on this topic.

The crosslinked gel has been produced by two partially different methods that both have their respective advantages and disadvantages. The freezing-thawing method (FT) is more limited in the range of initial chemical composition and the volume/thickness ratio that can be reliably produced and reproduced, while the gelation plus crosslinking at room temperature (RT) allows for more freedom with both. But freezing-thawing requires only a small amount of acid catalyst. The slow initial gelation rate is also convenient for the application of the mixture to the chosen supporting surface. The room temperature method on the other hand requires amounts of concentrated acid catalyst almost equal to those of the crosslinker. Significant parts of the acid remain in the finished gel and can't usually be removed or neutralized easily. The initial gelation rate is much higher, so the application has to be conducted quickly. The completion of the process, especially at higher thickness of the cast, takes much longer, and the shrinkage and accompanying expression of water has to be taken into account.

Mainly independent of the composition the RT gel is dense in absence of a support (bulk gel) material and forms mostly unbroken layers on the fibrous support (filter paper). FT gel on the other hand shows a pock-marked landscape under the microscope, especially

so if freeze-dried before the analysis. For filtration purposes RT gel would therefore have to be treated with either some porogene or suitably perforated.

Lipases can be immobilized on both types easily using the same crosslinker as used for the gelation, in case of RT gel only after one complete drying though. The available ADH enzymes can't be immobilized in active form the same way, the residual acid catalyst probably plays an important role in that.

The main advantage of using the bifunctional aldehyde GDA is its easy applicability that allows the immobilization or crosslinking in a single step without the need for activation. But the effect of the (symmetrical) crosslinker is basically random, the immobilization is not very controlled [Chae 2000]. Possible side reactions (as depicted in Fig.4.2.1) are the unintended crosslinking of the enzyme units, loss of GDA through further crosslinking of the PVA carrier and the formation of multiple enzyme-carrier bonds with the risk of activity loss through distortion (lipases seem to be less affected by this though, cf. Fig.3.14.6.7). The bonds are on the other hand not stable enough to prevent a slight but measurable leaching.

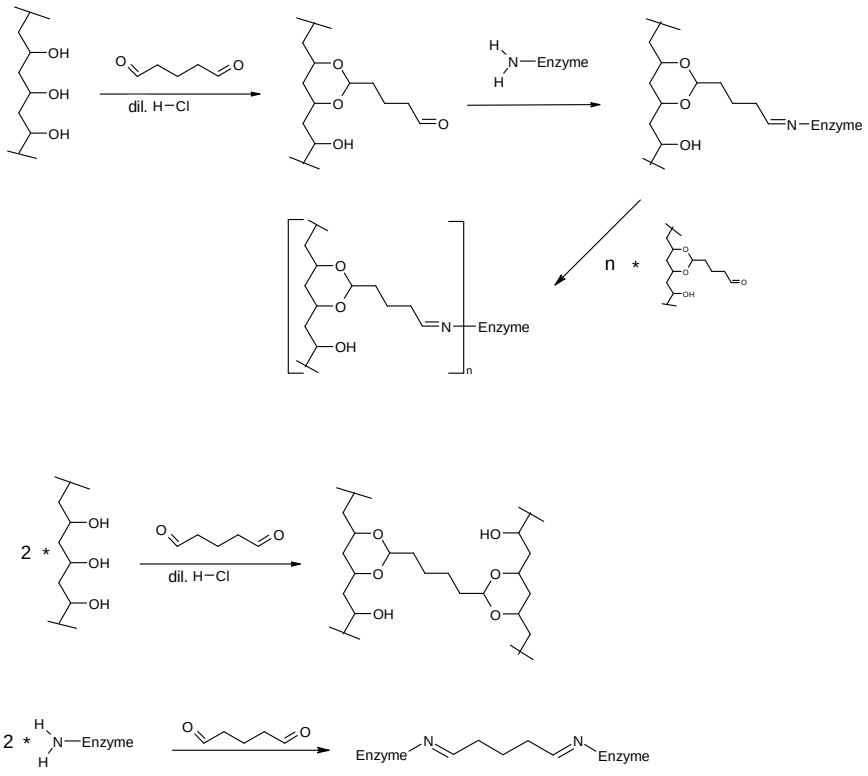


Fig.4.2.1: Immobilization of enzyme with GDA with possible undesired side reactions
The assumption of the formation of Schiff bases on the enzyme side is under dispute. A possible explanation for the observed deviating reactivity/stability is a subsequent internal rearrangement reaction[Tischer 1999].

These problem can probably be solved by the use of a different (and longer, [El-Zahab 2004]) anchor compound or changes in the fixation procedure. This is a very active field of research indeed, cf.e.g. [Yiu 2001], [Katchalski-Katzir 2000], [Tischer 1999]. Immobilization attempts during the gel formation process itself failed, the enzymes were completely deactivated. The thorough crosslinking also has the effect of preventing effective mass transport inside the gel, so only the surface can be used. That does not mean that successful encapsulation using this or similar materials are impossible per se. Restricting the gel stabilization to a thin surface layer (as with the LentiKat® procedure) seems to be a promising way. This includes also cofactor-dependent enzymes (e.g. [Ansorge-Schumacher 2002]).

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Berlin, the ____ of _____ 2008

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Signature

Appendices

Appendix A

Berkeley Madonna® programs for the simulation of the ADH/FDH process in Batch and CSTR reactors

Batch Reaktor with CPCR as primary enzyme

```
NADH0=100          ; NADH concentration at t =0 in µmol/l
NAD0=30            ; NAD concentration at t =0 in µmol/l
heptol0=0          ; Initial conc. of product in µmol/l
hept0=300          ; Initial conc. 300 µmol 34,2 mg
init NADH =NADH0
init heptol = heptol0 ;Initial conc. product
init hept = hept0          ; Initial conc. educt
NAD=NAD0                   ; def Initial conc. NAD
KiNADH=50                       ;µmol/l
KmNADH=1                        ;µmol/l
Kmhept=100                      ;µmol/l
Kihept=1000000                  ;µmol/l
kdesy=0.25/1440    ; 1/min Deactivation constant in min =(1,7*10^-4)
rmFDH=0.32         ;U/mg Reaction rate constant in min
rmADH=0.026        ;U/mg Reaction rate constant in min
FDH=130            ;FDH amount in mg/l
ADH0=130           ; ADH amount in mg/l
Kmform=200000      ;µmol/l
KmNAD=130 ;µmol/l
KiNADH=50          ;µmol/l
form=500           ;mmol/l Conc. formate
init ADH1=ADH0*0.63        ; 2 step deactivation kinetics
init ADH2=ADH0*0.37        ; 2 step deactivation kinetics
kdesy2=4.33/1440           ; 2 step deactivation kinetics 2nd parameter
d/dt(ADH1)=-0.63*kdesy*ADH     ; 2 step deactivation kinetics
d/dt(ADH2)=-0.37*kdesy2*ADH    ; 2 step deactivation kinetics
ADH=ADH1+ADH2
rADH=(rmADH*ADH)*(NADH/(KmNADH+NADH))*((hept)/(Kmhept*(1+(heptol)/Kihept)+h
ept)) ; 2. MM Gleichung Bildung von Heptanol mit ADH

rFDH=(rmFDH*FDH)*(form/(Kmform+form))*(NAD/(KmNAD*(1+NADH/KiNADH)+NAD)) ;
1. MM equation for regeneration of NADH with FDH

d/dt(hept)= -rADH
d/dt(heptol)= +rADH
d/dt(NADH)= -rADH +rFDH
NAD= NADH0-NADH
METHOD RK4
STARTTIME = 0
STOPTIME=10000
DT = 0.1
```

CSTR with HLADH as primary enzyme

```
VR= 100       ; Reaction volume
Flux= 0.01    ; Flow
Ret=0.95      ; Retention of NAD /NADH
Tau=VR/Flux           ; Residence time
TH=time/Tau           ; reduced residence time
NADH0=115*10^-3 ;mmol/l total amount
NAD0=0                ; NAD Concentration at t =0
heptol0=0             ; Initial conc. product
hept0=500             ; Initial conc. educt
init heptol = heptol0 ; Initial conc. product
init hept = hept0     ; Initial conc. educt
init NADH=NADH0
KiNADH=50*10^-3  ;mmol/l
rmADH=0.6*10^-3  ;U/mg Reaction rate constant in min
KmNADH=210*10^-3        ;mmol/l
Kmhept=612              ;mmol/l
Kihept=1000             ;mmol/l
kdesy=0.025/1440  ; Deactivation constant
rmFDH=0.32*10^-3        ;U/mg Reaction rate constant in min
FDH=5*3.6*10^-2  ;FDH amount in mg
Kmform=200              ;mmol/l
KmNAD=130*10^-3         ;mmol/l
KiNADH=50*10^-3  ;mmol/l
form=10                 ;mmol/l conz formate
init ADH = 10*5*10^-2 ; ADH Masse in mg
d/dt(ADH)=-kdesy*ADH ;Deactivation of the ADH 1 step model

rADH=((rmADH*ADH*1000)*(NADH/(KmNADH+NADH)))*((hept)/(Kmhept*(1+(heptol)
/Kihept)+hept)) ; 2. MM equation for formation of heptanol with ADH

hept0zul=500        ;Concentration heptanone in feed
heptol0zul=0 ;Concentration heptanol in feed
NADH0zul=0  ;Concentration NADH in feed
NAD0zul=0    ;Concentration NAD in feed

d/dt(hept)= 1/Tau*(hept0zul-hept) -rADH
d/dt(heptol)= 1/Tau*(heptol0zul-heptol)+rADH
d/dt(NADH)= -1/Tau*(NADH-Ret*NADH)
METHOD RK4
STARTTIME = 0
STOPTIME=100000
DT = 10
```

Appendix B

Table for scale dependent mixing of microemulsions

TDE7 [g]	C_6H_{12} [g]	H_2O [g]	m_{tot} [g]	w_0	Stirrer [min^{-1}]	Mixing time [s]
5	45	1.5	51.5	8.5	250	120 (1 Baffle) flakes remain
5	45	1.5	51.5	8.5	500	15-20(1 Baffle)
5	45	1.5	51.5	8.5	750	5(1 Baffle)
5	45	1.5	51.5	8.5	1000	8(1 Baffle)
5	45	1.5	51.5	8.5	1500	6(1 Baffle)
10	90	3	103	8.5	1500	10
10	90	3	103	8.5	1000	15
10	90	3	103	8.5	500	20-25 (near complete->complete)
10	90	3	193	8.5	250	120 (dregs, not affected by stirrer)
25	225	8	258	9.0	500	90-100
25	225	8	258	9.0	1000	35-40
25	225	8	258	9.0	1500	20
28	250	10	288	10.1	1500	20
35	327	11	373	8.9	1000	40-45
36	324	10	370	7.8	1500	15 (2 Baffles)
32	284	10	326	8.8	1500	30
51	460	15	526	8.3	250	>180 (strong flake-like dregs, not affected by stirrer)
51	460	15	526	8.3	500	120-150 (a few isolated flakes remain)
51	460	15	526	8.3	750	50
51	460	15	526	8.3	1500	30-35
81	723	24	828	8.4	1000	55(few isolated flakes)80(complete)
80	720	24	824	8.5	1500	15-20
81	724	24	829	8.4	2000	12-15
81	723	24	828	8.4	500	>180 (sticking on walls, flakes, turbid)
100	900	30	1030	8.5	1000	90-100(without baffles)
100	900	30	1030	8.5	1500	40-45(without baffles)
100	900	30	1030	8.5	2000	30(without baffles)
140	1260	42	1442	8.5	1000	120 (3 Baffles) (few isolated flakes, sticking on walls)
140	1260	42	1442	8.5	1500	40 (near complete)50-55(complete) (3 Baffles)
140	1260	42	1442	8.5	2000	30(near complete)35(complete) (3 Baffles)
211	1909	64	2184	8.6	1500	80-85(small flakes, strong sticking) (4 Baffles)
213	1917	64	2194	8.5	2000	35(near complete)45-50(complete) (4 Baffles) (sticking on lee side)
214	1926	65	2205	8.6	2000	45-50(no baffles)

Appendix C

Approximation for activity of generic ADH in a microemulsion

The γ-dependency shows an s-shape that can be approximated as follows:

$$A(\gamma) = (1/\pi) * \arctan [2*(\gamma-8.5)] + 0.5$$

Outside the area of microemulsion stability the activity is set as negligible and for γ values >13 the viscosity becomes too high to be of technical use.

The w-dependency shows a lower plateau and a peak that can be fitted polynomially:

$$A(w_0) = \sum_{i=0}^{6} a_i * x^i$$

with the following parameter values:

i	a_i
0	-22.67101
1	26.17313
2	-12.42001
3	3.09539
4	-0.42395
5	0.03016
6	$-8.68827*10^{-4}$

The total activity A_{tot} can be expressed as: $A_{tot} = A(w_0) * A(\gamma)$

in the area of about γ = ε[4;13] and w_0 = ε[3-10] as seen in Fig.App.C.1

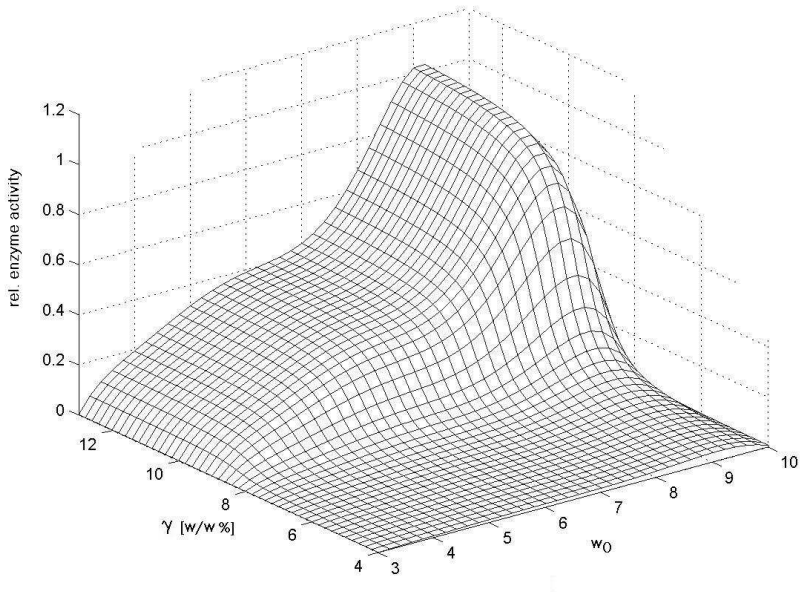

Fig.App C.1: generic ADH activity profile in w/o microemulsions