Pia Müller

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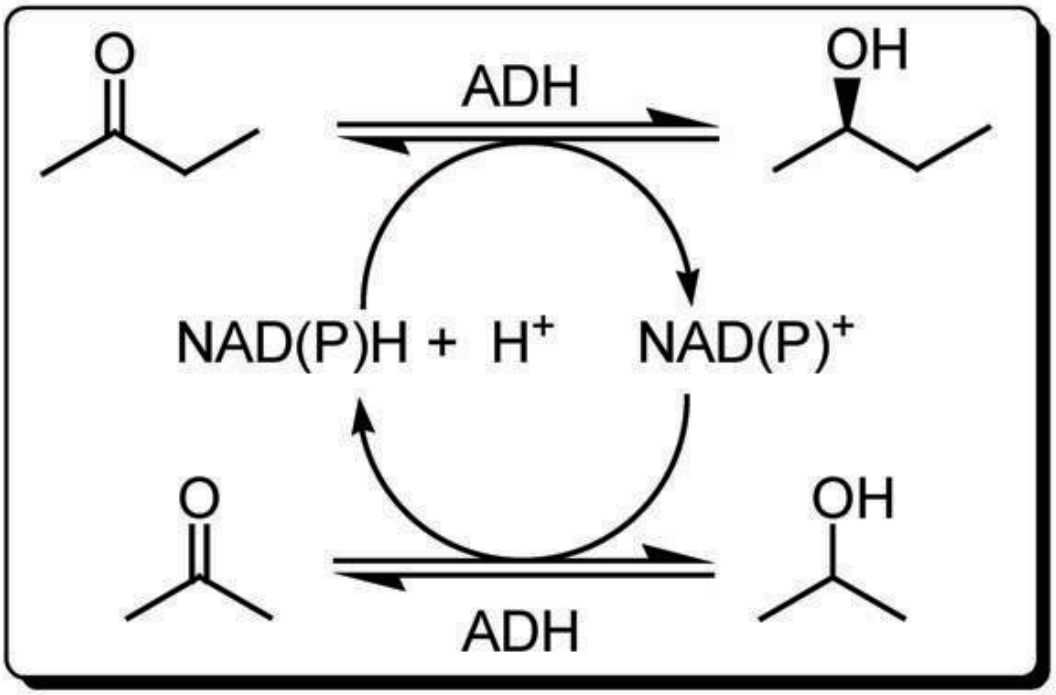

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Enantioselective Enzymatic Reduction of Prochiral Ketones in One-Phase and Two-Phase Systems

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Abstract

Within this thesis a strategy for the enantioselective synthesis of chiral short chain alcohols using biocatalysis is developed. This is realized in close collaboration with an industrial partner. The biocatalysts used are alcohol dehydrogenases (ADH) which need nicotinamide cofactors (NAD^+/NADH and $NADP^+$/NADPH) as redox equivalents. The regeneration of the cofactors is done substrate dependent by addition of 2-propanol which is oxidized by the ADH while the cofactor is reduced, and enzyme dependent. Here, a malate dehydrogenase (MDH) as second enzyme and *L*-malic acid as specific substrate are used for *in situ* cofactor reduction.

The kinetic characterization of the different ADH preparations and of a NAD- and a NADP-dependent MDH shows strong dependence of activity on the substrate, on reaction parameters like concentrations of buffer, substrate, and cofactor, on the type of ADH and on the kind of preparation, i.e. lyophilized or purified. The obtained results are transferred to one-phase batch synthesis of (*R*)- and (*S*)-2-butanol. Conversion and enantioselectivity (*ee*) are dependent on reaction conditions, i.e. 2-propanol and substrate concentration. Generally it is possible to synthesis both enantiomers of 2-butanol in the one-phase system. Due to limitations like low conversion, low selectivity, and low substrate solubility the synthesis of (*R*)-2-butanol is transferred to two-phase reaction systems using methyl-*tert*-butyl ether (MTBE) and the tailor-made ionic liquid (IL) as non-reactive phase. MTBE turns out to be the solvent of choice. Depending on substrate and co-substrate concentration conversion and *ee* can be positively influenced. The same is found for two-phase reactions with MTBE in a continuous reaction set-up. With the optimum reaction conditions obtained from the batch experiments conversion and *ee* are improved. Another influencing factor in the continuous reaction is the flow rate. ADH and cofactor show exceptionally high stability and high TTN. Together with further development of a work-up strategy the continuous two-phase reaction set-up will be a strong tool to produce enantiopure alcohols on preparative relevant scale.

Im Rahmen dieser Arbeit wird eine Strategie zur enzymatischen Synthese niedermolekularer enantiomerenreiner Alkohole entwickelt. Dies geschieht in enger Zusammenarbeit mit einem Industriepartner, der verantwortlich für die Entwicklung der Biokatalysatoren ist. Bei den verwendeten Enzymen handelt es sich um Alkoholdehydrogenasen (ADH), die Nicotinamidcofaktoren (NAD^+/NADH and $NADP^+$/NADPH) als Redoxäquivalente benötigen. Die Regenerierung der Cofaktoren wird sowohl substratgekoppelt unter Einsatz von 2-Propanol, welches von der ADH unter Reduktion des Cofaktors oxidiert wird, als auch enzymgekoppelt realisiert. Hier werden eine Malatdehydrogenase (MDH) als zweites Enzym und *L*-Äpfelsäure als spezifisches Substrat für die Cofaktorregenerierung verwendet.
Die kinetische Charakterisierung der verschiedenen ADH-Präparationen, der NAD- und der NADP-abhängigen MDH zeigt eine deutliche Abhängigkeit der Enzymaktivität vom Substrat, von Reaktionsparametern wie Puffer-, Substrat- und Cofaktorkonzentration und von der Art der ADH bzw. der Art der Präparation. Die erhaltenen Ergebnisse werden in ein Einphasensystem zur Batchsynthese von (*R*)- und (*S*)-2-Butanol übertragen. Umsatz und Enantioselektivität (*ee*) zeigen eine Abhängigkeit von der 2-Propanol- und der Substratkonzentration. Im Einphasensystem treten Limitierungen wie niedriger Umsatz, niedriger *ee* und schlechte Substratlöslichkeit auf. Generell ist es möglich Synthesen im Einphasensystem durchzuführen. Aufgrund der Limitierungen werden die gewonnenen Ergebnisse für die Synthese von (*R*)-2-Butanol in Zweiphasensysteme mit Methyl-*tert*-butylether (MTBE) und einer ionischen Flüssigkeit (IL) als nicht-reaktive Phase übertragen. MTBE erweist sich als Lösungsmittel der Wahl. Abhängig von Substrat- und Cosubstratkonzentration können Umsatz und *ee* positiv beeinflusst werden. Des Weiteren zeigt sich eine deutliche Verbesserung gegenüber dem Einphasensystem. Übertragen des Systems in einen kontinuierlich betriebenen Zweiphasenreaktor führt zu einer weiteren Verbesserung von Umsatz und *ee* in Abhängigkeit von der Verweilzeit. ADH und Cofaktor weisen sehr gute Stabilität und hohe TTN auf. Zusammen mit der Entwicklung einer entsprechenden Strategie für die Aufarbeitung handelt es sich bei dem kontinuierlichen zweiphasigen Reaktionssystem um einen zuverlässigen Prozess zur Produktion enantiomerenreiner Alkohole im präparativen Maßstab.

Die vorliegende Arbeit wurde in der Zeit von September 2005 bis März 2009 am Institut für Technische und Makromolekulare Chemie der RWTH Aachen University unter Leitung von Prof. Dr. Walter Leitner angefertigt.

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I Table of Contents

II List of Symbols, Abbreviations, and Acronyms

[BMIM][$(CF_3SO_2)_2N$]	1-butyl-3-methylimidazolium-bis(trifluoromethansulfon)imid
[BMIM][PF_6]	1-butyl-3-methylimidazolium-hexafluorophosphate
[BMIM][TFA]	1-butyl-3-methylimidazolium-tetrafluoro-acetate
[PMIM][PF_6]	1-pentyl-3-methylimidazolium-hexafluorophosphate
2-HexO	2-hexanone
A	area
ADH	alcohol dehydrogenase
AP	acetophenone
b_p	boiling point
BuO	2-butanone
BuOH	2-butanol
c	concentration
c_S	substrate concentration
*CP*CR	carbonyl reductase from *Candida parapsilosis*
CSTR	continuously stirred tank reactor
DSP	down-stream processing
E. coli	*Escherichia coli*
ee	enantiomeric excess
exp.	experiment
FDH	FDH
GC	gas chromatography
GDH	glucose dehydrogenase
h	hour
HL-ADH	horse liver alcohol dehydrogenase
IL	ionic liquid
K_M	Michaelis-Menten constant
K_S	substrate excess inhibition constant
k_x	reaction constant
LB-ADH	*Lactobacillus brevis* alcohol dehydrogenase
LK-ADH	*Lactobacillus kefir* alcohol dehydrogenase
log P	logarithm of partition coefficient of a compound in the standard n octanol/water biphasic system
LS-ADH	*Leifsonia sp.* alcohol dehydrogenase
m	slope of linear function
max	maximum
MDH	malate dehydrogenase
MTBE	methyl *tert*-butyl ether
NAD^+	nicotinamide adeninedinucleotide (oxidized form)
NADH	nicotinamide adeninedinucleotide (reduced form)
$NADP^+$	nicotinamide adeninedinucleotide phosphate (oxidized form)
NADPH	nicotinamide adeninedinucleotide phosphate (reduced form)
PF-ADH	*Pseudomonas fluorescens* alcohol dehydrogenase
P_X	partition coefficient of compound X

rac	racemic
S	substrate
SADH-X2	(*S*)-selective alcohol dehydrogenase
scCO_2	supercritical carbon dioxide
STY	space-time yield
subst.	substrate
t	time
T	temperature
τ	residence time
TTN	total turnover number
UV/Vis	ultraviolet/visible
V	volume
v_0	initial reaction rate
v_{max}	maximum reaction rate

1 Introduction

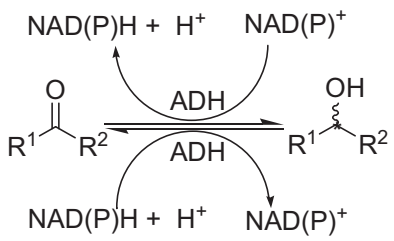
1.1 Biocatalysis using Alcohol Dehydrogenases

Enantiopure short-chain alcohols are important chiral building blocks for pharmaceuticals. They are used as chiral auxiliars and generally serve as intermediates for enantiopure substances. Furthermore, depending on the enantiomer chiral short-chain alcohols have a high added value compared to the prochiral ketones they are derived from (Table 1-1). For example, (*R*)-2-butanol is by a factor of about 120 more expensive than 2-butanone.[1, 2]

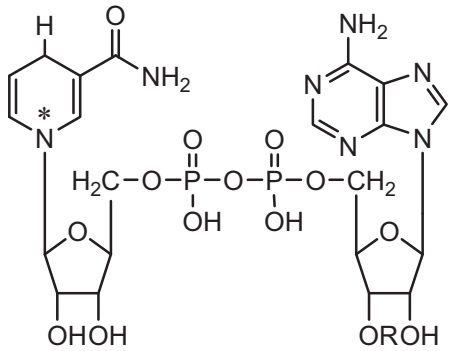
Table 1-1 Prices of ketones and corresponding (*R*)- and (*S*)-alcohols[3]

	price / € mol^{-1} ketone	price / € mol^{-1} (*R*)-alcohol	added value (*R*)	price / € mol^{-1} (*S*)-alcohol	added value (*S*)
2-butanone	28	3294	119	641	23
2-pentanone	121	29710	245	24594	203
2-hexanone	47	19299	410	12056	256
2-heptanone	124	19343	156	10609	86
2-octanone	12	5629	488	3800	329
acetophenone	82	1869	23	1112	14
4-chloro acetophenone	17	Merck on request	-	12453	737

Alcohol dehydrogenases (ADH) belong to enzyme class E.C. 1.1.1. They oxidize alcohols to the corresponding ketones or aldehydes, and reduce aldehydes and ketones to the corresponding alcohols (Scheme 1-1). Depending on the reaction they need the nicotinamide cofactors (Scheme 1-2) NAD(P)$^+$ (for oxidation reaction) or NAD(P)H (for reduction reaction) as hydride acceptor or hydride source.



Scheme 1-1 Scheme of ADH catalyzed reaction



NADH: R = H
NADPH: R = PO_3^{2-}

Scheme 1-2 Reduced forms of nicotinamide cofactors

Enzymes offen exhibit certain advances for asymmetric catalysis compared to chemical catalysis. Especially for difficult reactions biocatalysts are a worthful addition to chemical catalysts since even for difficult reactions biocatalysts can lead to excellent enantiomeric excess (for detailed examples see pp 3). Enzymes exhibit high regio-, chemo-, and enantioselectivity.[4] Furthermore, enzyme catalyzed reactions are usually possible under mild reaction conditions, i.e. moderate temperatures, nearly neutral pH values, aqueous medium, and an inert atmosphere is not necessary. Side reactions like isomerization, racemization, and epimerization of the products can be reduced. Due to difficulties with enantiotopic differentiation, chemical catalysis is hardly suitable for the production of enantiopure alcohols from prochiral ketones with very similar R-groups. 2-butanone is the smallest prochiral ketone. Its R-groups methyl and ethyl are very similar. Therefore, the enantiotopic differentiation is based on a very low energy difference leading to low enantioselectivities when using chemical catalysis. Besides these advantages ADH catalyzed reactions also have some disadvantages:

- low stability of ADH
- expensive and instable cofactors
- low water-solubility of substrates and products
- down-stream processing of aqueous reaction systems (expensive product isolation)
- thermodynamic limitation

Both, ADH and nicotinamide cofactors are instable in presence of high concentrations of organic solvents. Nicotinamide cofactors are especially instable in their reduced form. Thus, the stoichiometrical need of those nicotinamide cofactors is disadvantageous. Therefore, they are introduced to the reaction substoichiometrically, and different methods of cofactor

regeneration are employed. The most common ones are substrate and enzyme dependent cofactor regeneration. For the substrate dependent method a second cheap alcohol, e.g. 2-propanol, is added to the reaction mixture. This alcohol is oxidized while the cofactor is reduced by the ADH used for the alcohol production. When performing enzyme dependent cofactor regeneration a second enzyme, e.g. a glucose dehydrogenase (GDH) or a formate dehydrogenase (FDH), and a specific substrate, e.g. glucose or formate, are used. The second enzyme oxidizes its specific substrate while regenerating the cofactor.

Due to the aqueous medium the choice of substrates is limited to water-soluble compounds, and substrate concentrations are limited. Low substrate solubility can be overcome by employing co-solvents,[5, 6] or by using biphasic reaction systems.[7-9] Here, an water-immiscible organic solvent or an ionic liquid (IL) which is not miscible with water is used as non-reactive phase, i.e. as substrate reservoir and as extraction medium for the products. The catalyst and the cofactor remain in the reactive phase, i.e. aqueous phase. Higher substrate concentrations and poorly water-soluble substrates can be employed. Another disadvantage of reactions catalyzed by ADH is the isolation of the product from the reaction mixture. Due to the number of compounds present in the mixture, i.e. substrate, product, co-substrate, co-product, distillation from the mixture or extraction and subsequent distillation are hard to realize with quantitative product recovery. Since biocatalytic processes in general tend to have low productivities, a continuous setup in one-phase as well as in two-phase systems is desirable to enlarge productivity by continuous substrate dosage and integrated work-up.

Despite these difficulties biotransformations have become an accepted method for the economic production of short chain chiral alcohols for fine chemicals.[1, 10] Isolated ADH are industrially used for production of fine chemicals as well as whole cells.[10-15] Further interdisciplinary development in this field is expected to foster the scope and applicability of biocatalytic processes.

The typical reaction systems in which ADHs are employed are aqueous one-phase systems. Comparison of enzymatically and chemically catalyzed reduction of prochiral ketones reveals that with enzyme catalysis it is possible to achieve better chemo- and enantioselectivity for practically relevant products. For the reduction of the smallest prochiral ketone 2-butanone to the enantiopure (*R*)- and (*S*)-alcohol the highest reported *ee* using a chemical catalyst is 72% up to now. With a biocatalytic reaction it was possible to derive an *ee* of 99%.[16, 17]

Hummel reported on a NADP-dependent ADH isolated from *Lactobacillus kefir* (LK-ADH) which was one of the first ADH able to reduce acetophenone.[18, 19] The enzyme is highly (*R*)-specific, it does not form the (*S*)-alcohol at all or accept it as substrate in the alcohol oxidation. The reaction is carried out continuously in an enzyme-membrane reactor and with

enzyme dependent cofactor regeneration using glucose-6-phosphate dehydrogenase and glucose-6-phosphate. After 52 h a conversion of 84% was measured at an *ee* of >99%. Parallel investigations by Hummel *et al.* on an ADH from *Thermoanaerobium brockii* which is known for its thermal stability and broad substrate range showed that it is not able to catalyze the reduction of bulky phenyl-alkyl ketones like acetophenone.[19] Further investigations on the first mentioned LK-ADH allowed for stabilization of the enzyme by addition of Mg^{2+} ions and showed enzyme activity in presence of a large number of phenyl-alkyl and long-chain alkyl-alkyl ketones.[20] Best results of 86% conversion were obtained using the approved glucose-6-phosphate dehydrogenase/glucose-6-phosphate system for cofactor regeneration.
Acetophenone became the favored model substrate and was used for many biotransformations with whole cells and isolated enzymes. E.g. Matsuda *et al*. compared the reduction of acetophenone using either whole cells of *Geotrichum candidum* or an (*R*)-selective or an (*S*)-selective isolated ADH from this organism.[21] The reactions were carried out using either an excess of NADPH or $NADP^+$ and cyclopentanol for substrate dependent cofactor regeneration. Depending on the chosen conditions yields between 0% and 86% where achieved while the *ee* is settled between 22% and >99% (*S*) or (*R*), respectively.
More publications reported about new wild-type and recombinant ADH from different species. Hildebrandt *et al*. used a recombinant NAD-dependent ADH from *Pseudomonas fluorescens* (PF-ADH) for the reduction of acetophenone and its derivatives to (*R*)-alcohols.[22] *Ee* and conversion showed different optimum temperatures. Two maxima in *ee* of >99% were found at 10 °C and at 40 °C while conversion had its maximum of 95% at 20 °C. The same was valid for the dependence of selectivity and conversion on the concentration of 2-propanol which was used for cofactor regeneration. Highest conversion was found at 20% (v/v). Best enantioselectivity of >99% was achieved between 35% and 40% (v/v).
The necessity of optimization of reaction conditions was also investigated by Inoue *et al*.[23] The isolated ADH from *Leifsonia* sp. S749 (LS-ADH) showed a broad substrate range including 2-pentanone as smallest prochiral ketone. Most of the substrates were converted to (*R*)-alcohols with *ee* >99% including 2-pentanone and acetophenone. Reaction conditions were optimized for the conversion of 2,2,2-trifluoro acetophenone regarding concentrations of 2-propanol and NAD^+, and pH. At optimized conditions nearly quantitative conversion was observed.
Zhu *et al*. also reported on the dependence of enantioselectivity and activity of ADH catalyzed reactions on reaction conditions.[24] Increasing temperature enhanced enzyme activity while enantioselectivity was not influenced by temperature changes. Furthermore, the type of substrate was important for enantioselectivity. The ADH had a high tolerance of water-miscible and water-immiscible organic solvents. This is important to carry out the

reduction of hydrophobic ketones since co-solvents or a two-phase reaction system can be employed. For an ADH from *Saccharomyces cerevisiae* it could also be proven that the stereospecificity is dependent on the type of the prochiral ketones.[25]

Besides reaction conditions the 2'-, 3'-, and 4'-substituents of acetophenone derivatives play an important role for conversion and enantioselectivity.[26] For one ADH the *ee* can vary between >99% (*R*) and >99% (*S*) depending on the acetophenone derivative. Most of the acetophenone derivatives are very well accepted by a range of selected enzymes. Acetophenone was one of the best accepted substrates. Further investigations with an ADH from *Sporobolomyces salmonicolor* revealed a strong dependence of activity and enantioselectivity on the 1-substituent of acetophenone.[27] Activity was settled between fair to good values while the *ee* was either (*R*) or (*S*). Investigations on enzyme-substrate docking mechanism showed that depending on the substrate different active sites of the ADH are employed in the reaction.[28]

Since not all ADH show high stability in continuous processes Findrik *et al*. developed a mathematical model to describe the deactivation of an ADH from *Thermoanaerobacter* sp. in a batch, repetitive-batch, and continuously stirred tank reactor (CSTR). As model reaction the reduction of acetophenone was chosen.[29] The investigations showed an increase in enzyme activity with temperature. Furthermore, it is inhibited by the substrate acetophenone and the co-substrate 2-propanol and by all reaction products, i.e. (*S*)-1-phenylethanol and acetone. Fast deactivation was found in the CSTR and repetitive batch system. It could not be overcome by a change in reactor type. The developed mathematical model described the behavior of the reaction system very well and showed that mathematical descriptions can be useful to understand and predict the behavior of enzymatic reaction systems.

The immobilization of an ADH form *Lactobacillus brevis* (LB-ADH) on an amino-epoxy support enabled the continuous reduction of acetophenone with substrate dependent cofactor regeneration in a plug-flow reactor for 10 weeks without any significant loss in enzyme stability.[30]

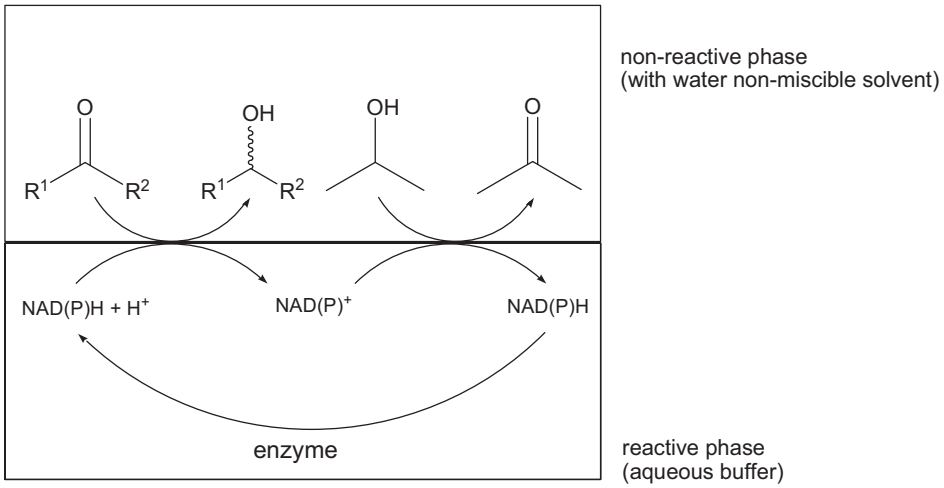
The most challenging products derived from a ketone reduction with a biocatalyst are (*S*)- and (*R*)-2-butanol as the corresponding ketone 2-butanone is the smallest prochiral ketone. Often, the reduction of 2-butanone resulted in racemic mixtures or in poor *ee*, but good conversions were possible.[31] Hochuli *et al*. reported on an ADH from *Mucor javanicus* able to enantioselectively reduce prochiral ketones.[32] The reduction of 2-butanone resulted in a racemic mixture while for 2-pentanone, 2-hexanone, and 2-octanone *ee* >99% were obtained. The energy difference for the enantiotopic differentiation between the two R-groups of 2-butanone was too low, and the necessary hydrophobic interaction in the active site could not be created by 2-butanone. With a thermostable and organic solvent tolerating ADH from

Thermoanaerobium brockii it was possible to achieve an *ee* of 48% pro-(*R*) for the reduction of 2-butanone. With growing cells of the organism *ee* was only 12%.[33] Shen *et al*. isolated a (*R*)-selective ADH from a *Pseudomonas* species which allowed for an *ee* of 87% when reducing 2-butanone.[34] The ADH proved to be stable at the applied reaction conditions and lead to a higher *ee* than the thermostable ADH from *Thermoanaerobium brockii*.
Kazuoka *et al*. investigated the influence of reaction conditions on the activity of a cold-active and thermostable ADH isolated from *Flavobacterium frigidimaris*.[35] The results showed a clear dependence of enzyme activity and stability on temperature and pH value. Furthermore, activity was negatively influenced by several inhibitors like $MnCl_2$, $MgCl_2$, and $ZnCl_2$ which are known to positively influence activity of other ADH. The enzyme is active towards 2-butanone and allowed for enantioselectivities of up to 97% in gas-phase reactions depending on water activity.[36]
Besides isolated enzymes whole cells were used for the reduction of acetophenone as model substrate and for 2-butanone. Since one of the first attempts for the reduction of 2-butanone had been made with ≤8% conversion using whole cells of several *Pseudomonas* species[37] many new organisms were found for the production of chiral alcohols. Enantioselectivity varied depending on the substrate. E.g. for acetophenone with *Daucus carota* root yield was 73% at an *ee* of 92% while the yield for 2-butanone reduction was only 38% with an *ee* of 87%.[38] With whole cells of *Geotrichum candidum* yield and enantioselectivity of acetophenone reduction were greatly enhanced by addition of Amberlite XAD-7 as solid organic solvent.[39] Best yield of 94% was achieved under argon atmosphere without XAD-7 while the best *ee* of >99% was found under argon atmosphere in presence of XAD-7. Depending on the cofactor NAD^+ or $NADP^+$ and on the alcohol added for cofactor regeneration (2-propanol or cyclopentanol) the acetone powder of *Geotrichum candidum* APG4 enabled good yield and 76-94% *ee* for the reduction of 2-butanone to (*S*)-2-butanol. Resting cells of *Sulfolobus solfataricus* converted 45% 2-butanone to (*R*)-2-butanol with an *ee* of 35% whereas acetophenone was hardly converted.[40]

Wandrey *et al*. conducted comparative experiments regarding conversion and *ee* for the chemical and biocatalytical continuous reduction of acetophenone to (*R*)- and (*S*)-1-phenylethanol.[41] In an enzyme membrane reactor acetophenone was reduced using a carbonyl reductase from *Candida parapsilosis* (*CP*CR). The chemical catalysis was realized using an oxazaborolidine catalyst. For the biotransformation an *ee* of 99% was achieved while the chemically catalyzed pathway resulted in 94% *ee*. In contrast, with 1400 g L^{-1} d^{-1} space-time yield (STY) was higher for the chemical catalysis, while 88 g L^{-1} d^{-1} was achieved for the biocatalytical reaction.

1.2 Biocatalysis with Alcohol Dehydrogenases using Two-Phase Systems

A further development for biocatalytic reactions described above are two-phase systems. They are helpful tools to overcome limitations like low substrate solubility in aqueous media and allow for integrated work-up.[42, 43] Biphasic reaction systems consist of a reactive aqueous phase and a non-reactive phase which serves as substrate reservoir and as extraction medium. The reaction takes place at the phase boundary. Enzymes and cofactors are immobilized in the reactive phase. Depending on the partition coefficients P_X (Equation 5-1) of substrates and products they distribute between the two phases (Scheme 1-3). Especially the very stable lipases are used in biphasic reaction systems as they work at low water activity and are very tolerant towards high concentrations of organic compounds. In contrast, ADH are generally much more sensitive towards non-aqueous compounds.[44-47]



Scheme 1-3 General scheme of a biphasic reaction system for reduction of ketones

It was found that organic media can generally influence enzyme activity due to specific interactions.[6] Furthermore, the presence of high amounts of organic solvents may lead to formation of a new conformation with higher activity.[48] For ADH encapsulation and structural changes of the enzyme in presence of organic solvents mostly leads to deactivation. Though, it is possible to employ ADH in biphasic reaction systems and achieve promising enantioselectivity and conversion. Many non-miscible solvents are potential non-reactive phases for biotransformations using ADH. An important role is played by the partition coefficients defining the distribution of an organic compound between the phases in a two-phase system.[49, 50]

Bradshaw *et al*. reported on a new ADH from *Pseudomonas* sp. showing high activity towards phenyl-alkyl ketones.[51] Acetophenone was reduced to the (*R*)-alcohol in a biphasic

reaction system using hexane as non-reactive phase. Enantioselectivity was 94% at a yield of 34%. The ADH showed good stability in presence of the organic layer. Similar investigations were conducted with an ADH from *Lactobacillus kefir*.[52] Depending on the ketones *ee* was 94->99% with yields between 15% and 71%. An ADH from *Rhodococcus erythropolis* and a FDH from *Candida biodinii* proved to be active and stable in a water/*n*-heptane biphasic system.[7] Even preparative relevant conversions were achieved.[8]
Besides organic solvents ionic liquids (IL) can be used as non-reactive phase.[47, 53-56] Ionic liquids are low-melting salts (b_p <100 °C) consisting of a large organic cation and an anion. Due to the number of possible combinations of cations with anions they are called "designer-solvents".[57] Besides their low melting point they are hardly volatile and not flammable. This makes them easy to handle. Cull *et al*. reported on the first successful replacement of organic solvents by IL in biphasic biocatalysis.[53] Major constraints due to flammability of organic solvents can be overcome by use of IL. Another concept is the employment of an IL for the enzyme phase. One of the most prominent IL is benzyl-methyl-imidazolium hexafluoro-phosphate [BMIM][PF_6]. As in contrast to organic solvents IL are not soluble in CO_2 products can easily be extracted using supercritical CO_2 ($scCO_2$).[58-60] Nearly quantitative recovery is possible. In 2001 Howarth *et al*. published the first use of an IL for a whole-cell biotransformation with immobilized baker's yeast.[61] While for some reductions the enantiomeric excess can be influenced positively, it was decreased for others in presence of the IL. Investigations on whole-cell catalysis by Pfründer *et al*. showed that in contrast to organic solvents IL do not lower the stability of the cells. Even an increase in membrane integrity was observed.[62]
Eckstein *et al*. compared a conventional biphasic system consisting of an aqueous buffer and methyl *tert*-butyl ether (MTBE) with one employing [BMIM][$(CF_3SO_2)_2N$] ([BMIM][TFA]).[9] Cofactor regeneration was realized substrate dependent. Therefore, the partition coefficients of acetone and 2-propanol were determined for both systems resulting in values of ~1 for the water/MTBE system illustrating equal distribution of both compounds. For the water/IL system P_X values of 0.4 for 2-propanol and of 2.0 for acetone were obtained displaying the very good extraction of acetone out of the aqueous phase. This is beneficial for the shift of equilibrium. The MTBE containing system allowed for a conversion of 61% in the reduction of 2-octanone using the LB-ADH while the reaction in the IL system resulted in 88% conversion. Furthermore, the reaction was faster in the system containing the IL. Enantioselectivity was >99% in both systems. Obviously, the inhibiting effect of acetone could be reduced by the removal of acetone from the reactive phase. But the LB-ADH was by a factor of 100 less stable in the water/IL system. The results show that it is important to take partition coefficients into account when choosing the non-reactive phase for a biphasic reaction system.

Investigations by Musa *et al*. on the use of IL in two-phase reaction systems compared to one-phase systems using co-solvents or biphasic systems employing organic solvents showed that the system choice depends not only on enzyme stability but also on the substrate.[63] As reported by Dreyer *et al*. the IL Ammoeng110™ can be used for enzyme purification and has a stabilizing effect on ADH.[64] Furthermore, improved conversion and yield were found.
Because ADH need water to remain stable during the reaction it is hardly possible to use isolated ADH in non-aqueous media. There exist several concepts to realize and describe the use of non-aqueous media with biocatalysts:

- immobilization of enzymes in the aqueous phase
- aqueous biphasic systems employing water-immiscible organic solvents or IL as non-reactive phase
- micro-aqueous solutions
- the log P-concept; log P is the logarithm of the partition coefficient of a compound in the standard *n*-octanol/water biphasic system.[65, 66]

Immobilized and pure horse-liver ADH (HL-ADH) were stable in hexane.[67] Whole cells showed good stability in organic solvents as well.[68, 69] Activities could be improved by immobilization.[70, 71] Another possibility to use ADH in organic media are micro-aqueous systems composed of ≤1% water ≥99% organic solvent.[65, 72] An ADH from *Rhodococcus ruber* overexpressed in *E. coli* had proven high operational stability in the reduction of prochiral ketones with simultaneous cofactor regeneration using 2-propanol.[73-78] Cells of *E. coli* containing the overexpressed ADH were used in various mono- and biphasic aqueous-organic solvent systems with hardly any loss in activation.[65] For the micro-aqueous system water-miscible organic solvents were used which led to complete deactivation of the ADH. Water-immiscible solvents showed a higher biocompatibility causing no deactivation. Even cell-free ADH was hardly deactivated. The hydrophobicity of the solvents described by log P was clearly correlated with enzyme activity and with damage of the cell membrane. Solvents with log $P_X > 2$ were highly biocompatible. Pfründer *et al*. employed whole cells of *Lactobacillus kefir* for ketone reduction in a biphasic reaction system.[62] A clear dependence of the cell membrane damage by the organic solvent on log P was found. In contrast, IL did not damage the cell membrane at all. The process with integrated cofactor regeneration using glucose allowed for higher productivity than other whole-cell biotransformations in IL without integrated cofactor regeneration.[61] Furthermore, the established IL process performs well compared with industrial biocatalytic processes.[10, 13] By linear free energy correlation it is possible to predict values for log P.[79] Comparison of the predicted log P values provides a

method of predicting separations and of choosing a solvent system. The question arouse whether log P is an adequate criterion to choose solvents for aqueous biphasic reaction systems. Vilella *et al*. investigated the correlation between log P and a suitable solvent and concluded that not only the polarity but also the functionality of the organic solvent describes the biocompatibility of solvents.[80]

ADH were successfully applied in two-phase reaction systems to synthesize chiral alcohols with high enantiomeric excess. The advantages compared to one-phase systems are obvious. Although there is still the possibility of further development and optimization, with suitable choice of the solvent for the non-reactive phase and sufficient enzyme stability and activity relevant product amounts can be synthesized.

1.3 Cofactor Regeneration

For biocatalytic processes using ADH the presence of NADH or NADPH as hydride source is important. Since these cofactors are instable and expensive especially in their reduced form (Figure 1-1), they are employed substoichiometrically and continuously regenerated within a process. This regeneration can be done substrate[9, 80-84] or enzyme dependent[8, 81-85] or by means of electrochemistry[84, 86-88] and chemical catalysis[81, 89]. The first two are state of the art and applied industrially. Due to the importance of adequate supply with reduced cofactor special attention is paid to its regeneration.

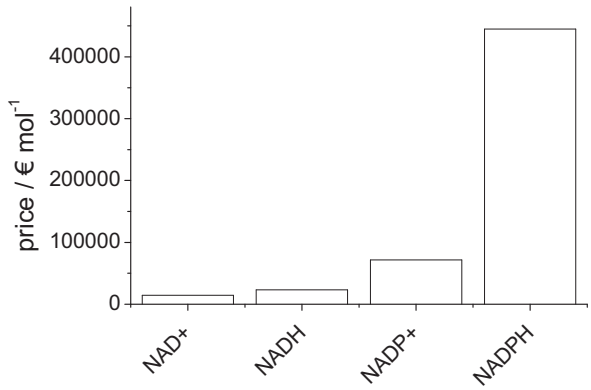


Figure 1-1 Prices for the nicotinamide cofactors (obtained from Carl Roth)[90]

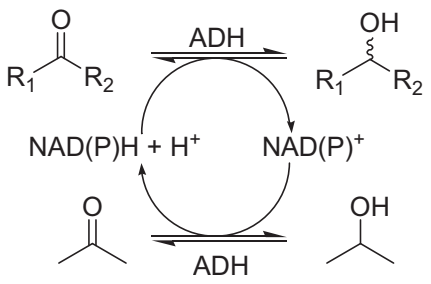
For substrate dependent cofactor regeneration the same ADH as for the reduction reaction and a second cheap alcohol, e.g. 2-propanol is used (Scheme 1-4). This co-substrate is

oxidized while the cofactor is reduced. The reversibility of this reaction, i.e. the reduction of the co-product acetone, has to be taken into account and influences the equilibrium (Equation 1-1) of the reaction.[91] By applying high 2-propanol concentrations equilibrium can be shifted to product side.[92-94] Another possibility according to Le Chatelier's Law is the continuous removal of the co-product acetone by pervaporation or by stripping.[91] If these methods are applied to the synthesis of (*S*)-5-hydroxy hexanoic acid ethylester conversion can be increased from 75% for the conventional system to 95% and >97%, respectively.[91]

Besides 2-propanol other secondary alcohols were tested for substrate dependent cofactor regeneration by Itoh *et al*.[95] For the ADH catalyzed synthesis of (*R*)-2-chloro-1-(3-chlorophenyl)ethanol the secondary C_4- to C_8-alcohols were tested for cofactor regeneration whereas 2-propanol gave the best results. Furthermore, the optimum pH for the reduction of phenylacetaldehyde and for the oxidation of 2-propanol by the same ADH was investigated. It was found that the optimum pH for the reduction is 6.2 while it is 10.5 for the oxidation. These results reveal that although only one enzyme is needed for both reactions it is challenging to find a good compromise between the optimum reaction conditions.

Nevertheless, 2-propanol is used for cofactor regeneration in industrial processes.[10] The complexity of the reaction system is reduced, only one enzyme is needed, and 2-propanol is an inexpensive reducing agent.

Substrate dependent regeneration systems are not only used in aqueous one-phase systems but also in gas-phase reactions[96] and in biphasic reaction systems.[83] Mostly, 2-propanol is used in excess to shift equilibrium. One of the first examples of enantioselective 2-butanone reduction in a one-phase system using 2-propanol as co-substrate was published by Keinan *et al*. in 1986.[33] A NADP-dependent ADH from *Thermoanaerobium brockii* was used resulting in an enantiomeric excess (*ee*) of 48% (*R*).



Scheme 1-4 Substrate coupled cofactor regeneration using 2-propanol

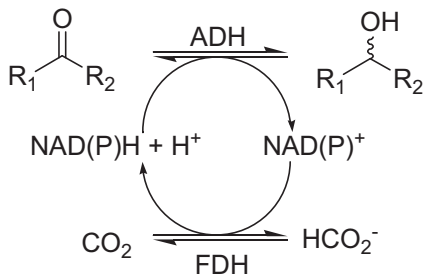
$$K = \frac{c_{product} \cdot c_{acetone}}{c_{substrate} \cdot c_{2\text{-propanol}}}$$

Equation 1-1 Equilibrium constant of ADH catalyzed reaction using substrate dependent cofactor regeneration

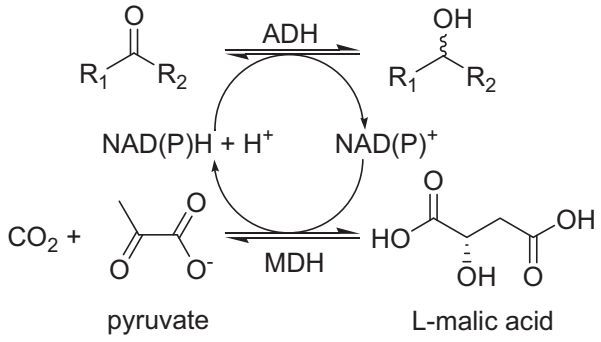
Enzyme dependent cofactor regeneration can be performed using different enzymes and their specific substrates:

- formate dehydrogenase (FDH), substrate is formate
- glucose dehydrogenase (GDH), substrate is glucose
- malate dehydrogenase (MDH), substrate is malate
- hydrogenase, substrate is molecular hydrogen

The most common ones are the FDH (Scheme 1-5) and the GDH (Scheme 1-7). The MDH has hardly been mentioned in literature[97] and has been recently introduced commercially (Scheme 1-6).[98] For all three methods the pH has to be monitored during the reaction because it will change with proceeding reaction. From the 1970s on NAD-dependent FDH were employed for cofactor regeneration.[7, 8, 81, 92, 99-102] Since the reactions were limited to NAD-dependent ADH attempts were made to identify and isolate a NADP-dependent FDH which was realized in 1996 by Seelbach *et al*.[85] The isolated mutant showed 40% less activity than the wild-type NAD-dependent FDH but has convincing stability. It is stable for seven days at 25 °C and can be stored at 4 °C for one year without any loss in activity. In a continuous reaction it allowed for 90-95% conversion. A TTN of 85 for $NADP^+$ was reached. The stability of a FDH could also be shown in two-phase systems.[7, 103] Although it is challenging to find optimum reaction conditions for the complex reaction system containing two different enzymes, FDH are used for cofactor regeneration in industrial processes.[10]

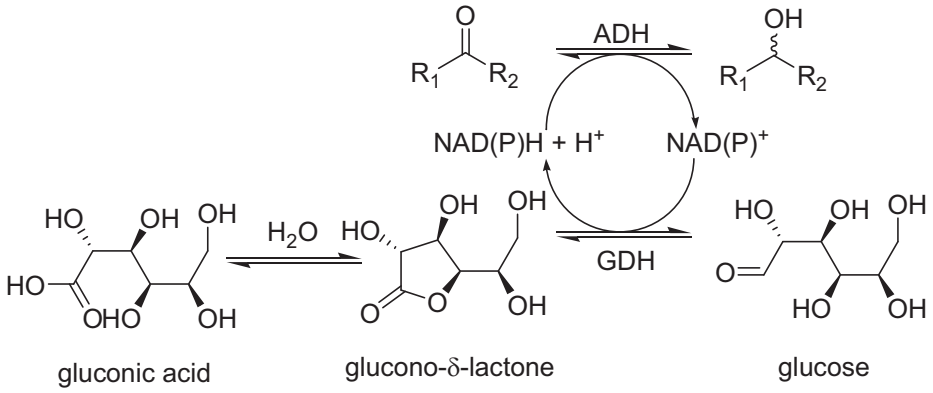


Scheme 1-5 Enzyme coupled cofactor regeneration using FDH and formate



Scheme 1-6 Enzyme coupled cofactor regeneration using MDH and *L*-malic acid

Another prominent example for enzymes used for enzyme dependent cofactor regeneration are GDH (Scheme 1-7). They can be used in one-phase[28, 81, 83] as well as in two-phase systems and in presence of high concentrations of organic solvents.[7, 24, 83, 103, 104] Favorable about GDH is that the co-product glucono-δ-lactone hydrolyses in aqueous media shifting the equilibrium to product side.[84] On the other hand the pH value changes due to formation of gluconic acid.



Scheme 1-7 Enzyme coupled cofactor regeneration using GDH and glucose

For the reduction of acetophenone to (*S*)-1-phenylethanol using an ADH from *Thermoanaerobium* species substrate and enzyme dependent cofactor regeneration using 2-propanol on the one hand and a hydrogenase from *Pyrococcus furiosus* and molecular hydrogen on the other hand were compared.[82] The reaction rate turned out to be higher when using the substrate coupled method (8.2 mmol h^{-1} vs. 4.0 mmol h^{-1}) while conversion was higher with the enzyme dependent regeneration (93% vs. >99%). The results lead to the conclusion that the substrate dependent regeneration is thermodynamically limited whereas the enzyme dependent route allows for quantitative conversion.

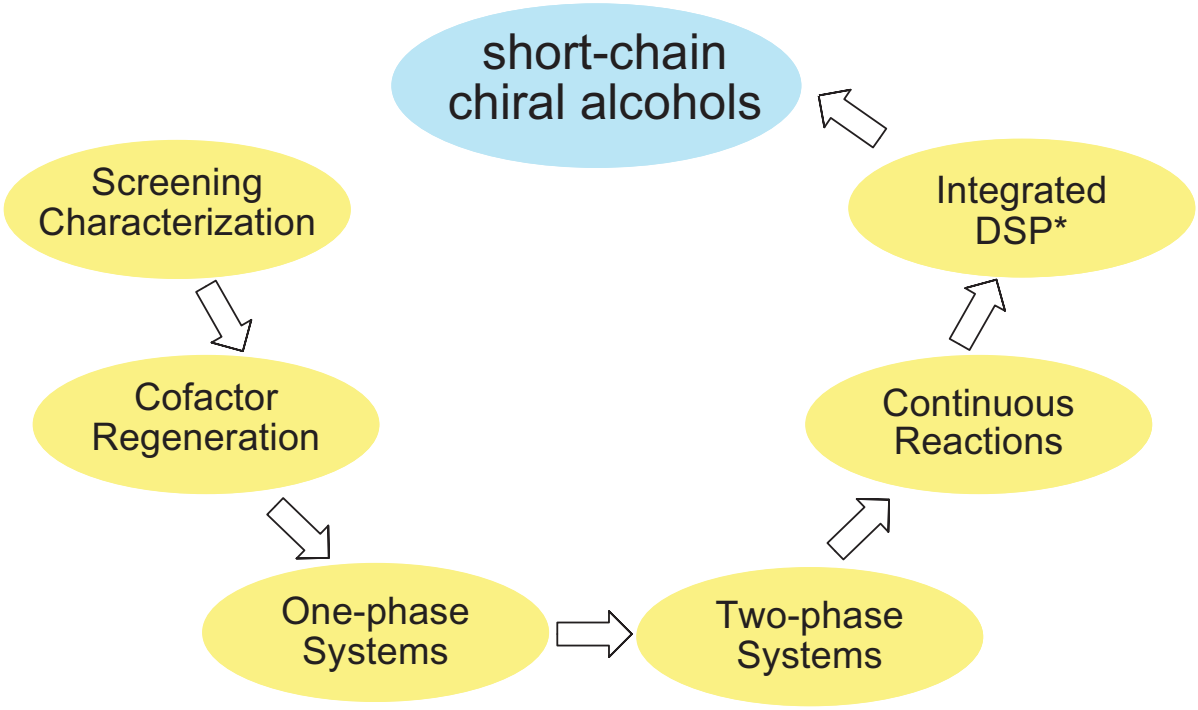
2 Motivation and Aim

Enantiopure short-chain alcohols are of high interest as intermediates for fine chemicals and pharmaceuticals. The added value compared to their corresponding ketones is exceptionally high (Table 1-1). Biocatalysis is a strong tool to provide high enantioselectivity for the synthesis of the desired product alcohols. The feasibility of their continuous synthesis using biocatalysis will be investigated in this work. Aiming for a two-phase system for the integration of down stream processing a staged approach will be pursued (
Figure 2-1). However, productivity, costs of the overall process and down-stream processing (DSP) are not yet developed to an extent to provide the compounds in sufficient quality and purity. Alcohol dehydrogenases produced by molecular microbiology methods, e.g. mutagenesis, are screened for their ability to reduce prochiral ketones enantioselectively to chiral alcohols. The choice of enzyme catalysis is due to the substrates of interest, i.e. short-chain prochiral ketones, for which sufficient enantiotopic differentiation is hard to achieve by chemical catalysis. Enzymes have already proven to be highly selective for the reduction of small ketones. It is known that reaction conditions strongly influence conversion and selectivity. It is therefore of key importance that the main reaction conditions influencing conversion and enantioselectivity are identified by screening.

First, screening experiments using different ADH will be carried out to show the dependence of enzyme activity on substrate and reaction conditions and to prove the impossibility to transfer optimum reaction conditions from one ADH to another (Chapter 3). The obtained results will be transferred to synthesis in one-phase systems to identify the main influence factors regarding conversion and enantioselectivity (Chapter 4). It will be shown that depending on the substrate/product pair racemisation occurs for which the mechanism will be investigated (Chapter 4.1.4). One-phase and two-phase systems will be used to prove the dependence of conversion and selectivity on reaction conditions and on the cofactor regeneration system. Two-phase systems will allow for overcoming problems with solubility, racemisation, and productivity (Chapter 5.2). Hardly water-miscible substrates can be employed and equilibrium is shifted to product side. The non-reactive phase serves as a feed phase and re-extracts the product out of the aqueous phase. Biphasic systems offer the opportunity for easy work-up which can also be integrated in a continuous process. The design of a continuous process will be based on the results obtained from the characterization and optimization of the one- and two-phase systems (Chapter 5.3).

Since the used ADHs are either NADH or NADPH dependent, also the regeneration of these cofactors is of relevance for the overall reaction. Here, both enzyme and substrate dependent approaches will be investigated for advantages and disadvantages. For the enzyme dependent regeneration the kinetic behavior of a NAD-dependent and of a

NADP-dependent MDH is investigated using UV/Vis spectroscopy (Chapter 3.5 and Chapter 3.5). Both MDH will be used for enzyme dependent cofactor regeneration in one-phase and two-phase systems (Chapter 4.1.2 and Chapter 5.2.1.2).



*DSP = down-stream processing

Figure 2-1 Stages of experimental work

3 Kinetic Characterization of Enzymes

3.1 Methodological Background

The pre-screening of enzymes by the industrial partner exhibited two ADH which are suitable for the enantioselective reduction of prochiral ketones, the NADP-dependent LB-ADH and the NAD-dependent SADH-X2. Of the LB-ADH two different preparations will be used: a standardized lyophilized preparation, and a liquid preparation which was specially purified by column chromatography. The SADH-X2 is also applied as standard lyophilized preparation. Furthermore, for enzyme dependent cofactor regeneration a lyophilized NAD-dependent and a lyophilized NADP-dependent MDH were pre-screened.

For the characterization of enzymes in general and ADH in particular kinetic parameters like maximum activity v_{max} and the Michaelis-Menten constant K_M are significant values. Furthermore, in case of substrate-excess inhibition the constant K_S is of importance. To perform kinetic measurements UV/Vis spectroscopy is applied. In case of an ADH characterization the absorbing species are the reduced cofactors NADH and NADPH. The change in absorption is direct proportional to the reaction rate. The change in absorbance is monitored at different substrate concentrations, i.e. ketone or alcohol, the initial reaction rate v_0 is calculated from it using the slope m (Figure 3-1, for more details see Chapter 7.5) and is plotted against the initial substrate concentration (Figure 3-2). This is the so-called Michaelis-Menten plot using Equation 3-1 providing two kinetic parameters, v_{max} (Equation 3-2) and K_M (Equation 3-3) based on the standard reaction scheme (Scheme 3-1). v_{max} is the maximum reachable reaction rate, K_M is the Michaelis-Menten constant describing the affinity of the enzyme towards the substrate at the given reaction conditions. A high value for the concentration at which ½ v_{max} is reached means a low affinity of the enzyme towards the substrate.

For the UV/Vis spectroscopy a multiplate reader by Biotek is used. The measurements are done using 96-well plates with a maximum volume of 220 µL. A maximum of 8 measurements is done in parallel. Reactions are usually started by addition of enzyme; measurements are started immediately after enzyme addition.

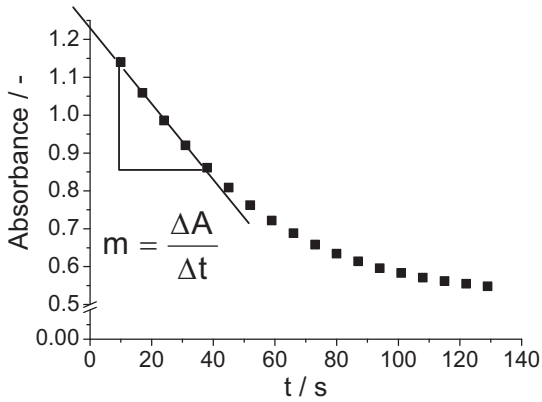


Figure 3-1 Illustration of typical absorption/time plot

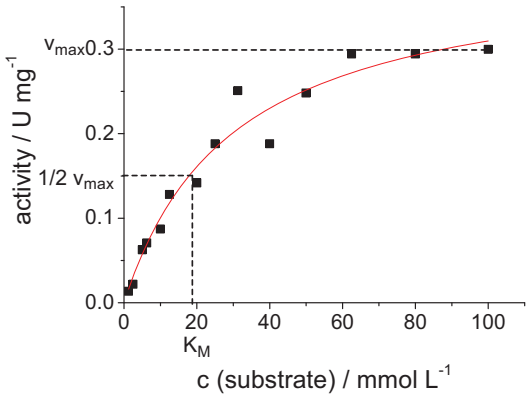


Figure 3-2 Illustration of typical Michaelis-Menten plot

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E+P$$

Scheme 3-1 General reaction scheme of an enzyme catalyzed reaction

$$v = \frac{v_{max} \cdot c_S}{K_M + c_S}$$

Equation 3-1 Michaelis-Menten equation

$$v_{max} = v \frac{K_M + c_S}{c_S}$$

Equation 3-2 Definition of v_{max} resulting from Michaelis-Menten equation

$$K_M = \frac{k_{-1} + k_2}{k_1}$$

Equation 3-3 Definition of K_M

The pre-screening of reaction conditions by the industrial partner showed the following standard conditions for the enzymatic assays for all three ADH preparations. Enzyme assays and the resulting activity data are the only way to characterize enzymes properly. As they are produced by means of molecular biology the protein content may vary which does not ensure continuous quality of activity.

c (cofactor) = 0.5 mmol L^{-1}
c (buffer) = 50 mmol L^{-1} potassium phosphate buffer
pH = 6.5
T = 30 °C

A pool of prochiral ketone substrates and acetone were selected for the screening as well as the corresponding alcohols (Figure 3-3). Substrate concentrations were chosen depending on the water-solubility of the respective substrate. Based on the standard assay the substrate spectrum of the three ADH will be tested. Furthermore, the dependence of the enzymes on the following parameters will be tested:

- buffer concentration
- cofactor concentration
- substrate concentration
- addition of salts ($MgCl_2$, $MnCl_2$)
- pH value
- saturation of the buffer with MTBE

The tolerance towards MTBE is important for the application of the enzymes in biphasic reaction systems. From the recorded data enzyme activity is calculated and plotted against the respective substrate concentration. If possible, a Michaelis-Menten fit is obtained using Origin. At the beginning of each sub-chapter all performed measurements including v_{max} and K_M are summarized to give an overview of the obtained results. The results show that every system reacts different to a change of a certain parameter. Optimum reaction conditions cannot be generalized.

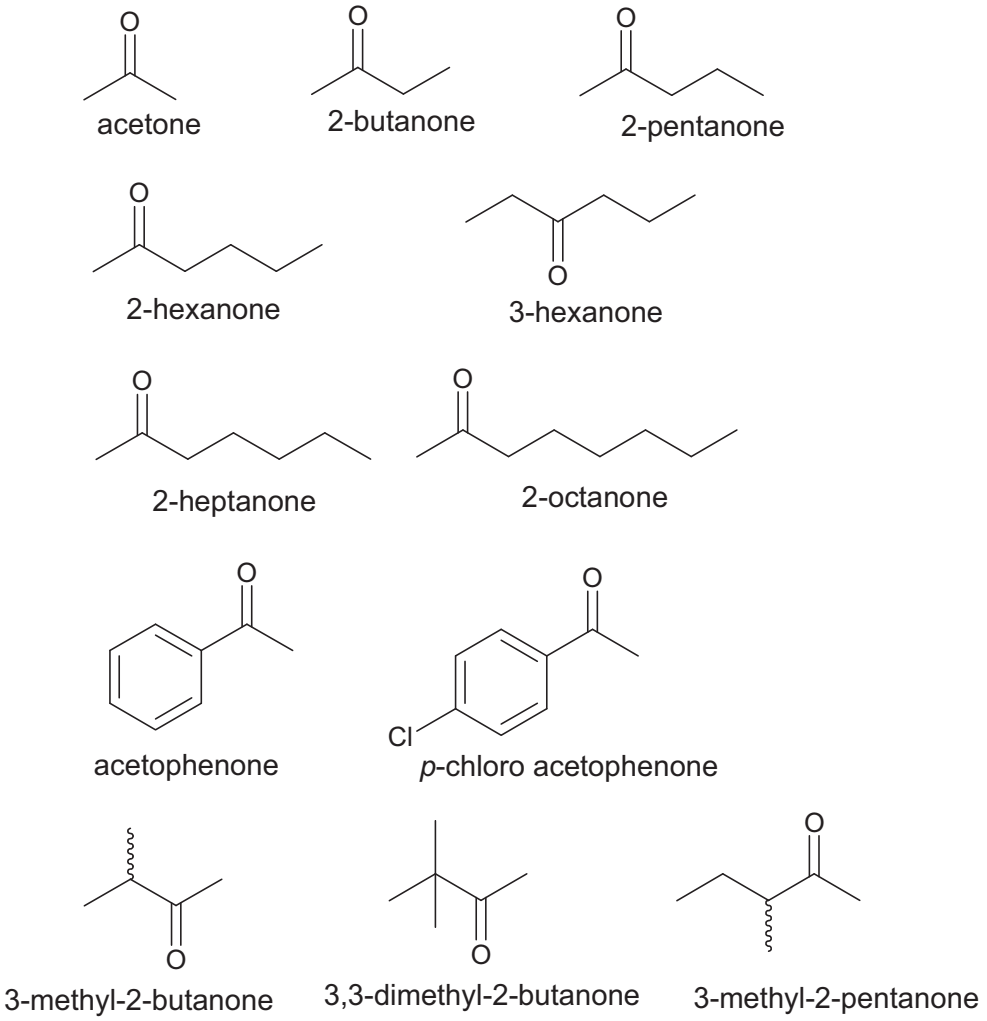


Figure 3-3 Prochiral ketone substrates

3.2 Substrate Screening

The solid SADH-X2, the solid and the liquid LB-ADH preparation are tested for their activity towards different substrates. All show good activities in presence of the selected ketones.

The SADH-X2 shows high activities of 12 – 14 U mg^{-1} for the reduction of 2-hexanone, 2-heptanone, and 2-octanone (Figure 3-4). The shorter the chain of the ketone is the lower is the activity. It increases from 0.5 U mg^{-1} for 2-butanone and 2.8 U mg^{-1} for 2-pentanone to 11.7 U mg^{-1} for 2-hexanone. The activity towards 3-hexanone is 7-times lower than towards 2-hexanone. Obviously, the ethyl group is too large to enter the active site of the enzyme. Acetone as a non-prochiral ketone is also accepted as a substrate, and the SADH-X2 has a larger activity in presence of this ketone than for the reduction of 2-butanone. The orientation of 2-butanone is not well defined although it has two different R-groups. The methyl and the ethyl group are very similar, but the ethyl group cannot enter the active site. Therefore,

activity towards 2-butanone is lower than towards acetone. Activity shows a certain dependence on the size of the non-methyl group of the secondary ketone. With increasing chain length the hydrophobic interactions with the enzyme increase leading to higher reaction rates.

The SADH-X2 is able to reduce aromatic and branched prochiral ketones as well (Figure 3-5). For acetophenone (4.7 U mg^{-1}) activity is lower than for *p*-chloro acetophenone (9.3 U mg^{-1}) due to electronic effects. The values are settled between those for 2-pentanone and 2-hexanone. For the branched ketones activities are lower than for aromatic ketones. Regarding the size of the R-groups the same trends as for the linear ketones can be found. The activity towards 3-methyl-2-butanone is 2.4 U mg^{-1} and, therefore, lower than towards 3-methyl-2-pentanone which provides a larger size difference in the R-groups. The sterically more demanding 3,3-dimethyl-2-butanone is too bulky to enter the active site although it contains a methyl group. Activity is only 1.5 U mg^{-1} which is 1.6-times lower than towards 3-methyl-2-butanone.

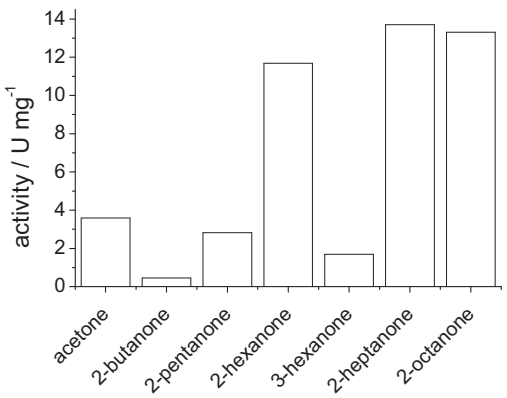


Figure 3-4 Activity of SADH-X2 towards linear ketones

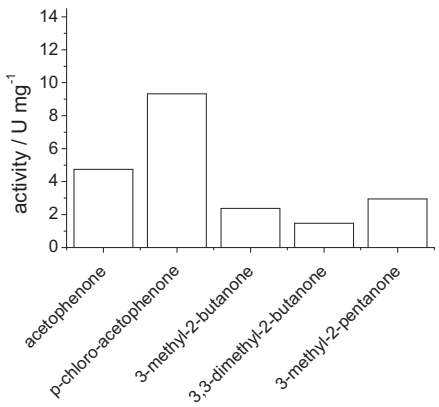


Figure 3-5 Activity of SADH-X2 towards bulky aliphatic and aromatic ketones

The solid LB-ADH preparation is tested for its activity towards linear, aromatic, and branched ketones. The activity towards 2-butanone is higher than towards acetone (Figure 3-6). The gap between the activity towards 2-pentanone (1.8 U mg^{-1}) and 2-hexanone (2.2 U mg^{-1}) is only small. For 2-heptanone a maximum in activity of 6.1 U mg^{-1} is reached. For 2-octanone activity is 4.6 U mg^{-1}.

As well as the SADH-X2 the solid LB-ADH shows activity for aromatic and branched ketones (Figure 3-7). Activity towards acetophenone is 4.7 U mg^{-1} and lower than towards *p*-chloro acetophenone with 8.2 U mg^{-1}. There is only a small difference of 0.3 U mg^{-1} obtained in activity for the reduction of 3-methyl-2-butanone (2.4 U mg^{-1}) and

3-methyl-2-pentanone (2.7 U mg^{-1}). In contrast to the SADH-X2 activity for 3,3-dimethyl-2-butanone is with 3.2 U mg^{-1} higher than towards the less bulky 3-methyl-2-butanone. The reason might be a larger active site of the LB-ADH compared to the SADH-X2.[105]

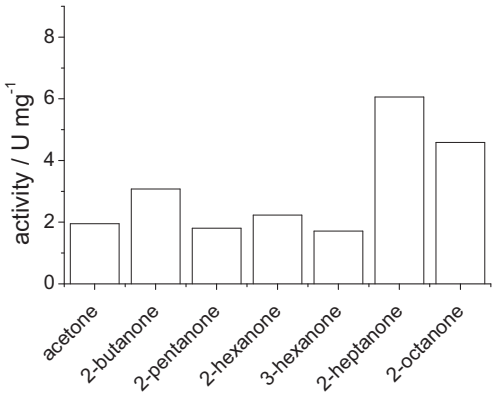


Figure 3-6 Activity of solid LB-ADH towards linear ketones

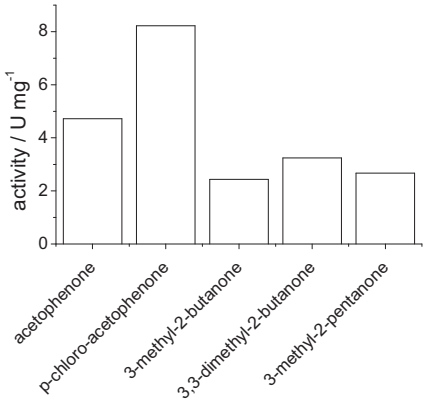


Figure 3-7 Activity of solid LB-ADH towards bulky aliphatic and aromatic ketones

The results obtained from measurements using the liquid LB-ADH preparation show that the preparation of the enzyme has a huge impact on activity. The liquid LB-ADH has high activity towards all tested ketones. The highest activity is observed for acetone with 606 U mL^{-1} (Figure 3-8). For this LB-ADH preparation the tendency of activity cannot be correlated to the chain length of the linear ketones. Surprisingly, the activity towards 3-hexanone is higher than towards 2-hexanone. This was neither found for the SADH-X2 nor for the solid LB-ADH preparation.

The activity measured towards acetophenone is 515 U mL^{-1} and lower than towards *p*-chloro acetophenone with 620.5 U mL^{-1} (Figure 3-9). For the bulky aliphatic ketones no tendency regarding the chain length is observed. Activity towards 3-methyl-2-butanone and 3,3-dimethyl-2-butanone is 369.3 U mL^{-1} and 385.1 U mL^{-1}, respectively. In contrast to the SADH-X2 and the solid LB-ADH the activity of the liquid LB-ADH towards 3-methyl-2-pentanone is lower than for the other two bulky ketones.

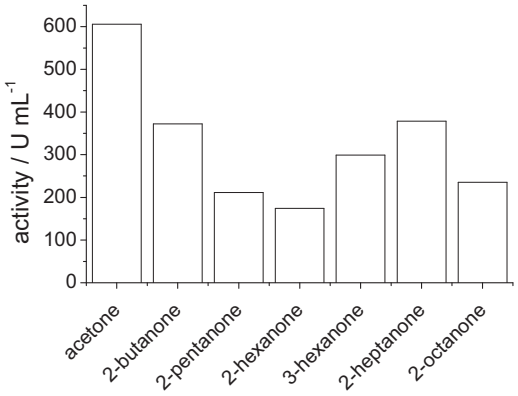


Figure 3-8 Activity of liquid LB-ADH towards linear ketones

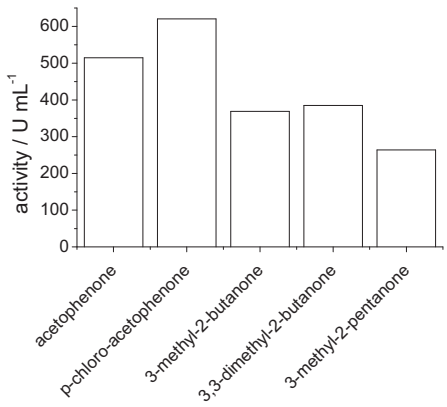


Figure 3-9 Activity of liquid LB-ADH towards bulky aliphatic and aromatic ketones

The substrate screening for the two different kinds of ADH and for the two different preparations of the same ADH reveals a high substrate spectrum for all three preparations. Furthermore, it is shown that it is impossible to predict the substrate spectrum and the activity towards different substrates. There is no possibility to infer from one enzyme to another or even to infer from one enzyme preparation of the same ADH to another preparation. This makes the characterization of each single enzyme essential for the creation of a process.

3.3 Kinetic Characterization of the SADH-X2

3.3.1 Introduction

From kinetic measurements for the characterization of the SADH-X2 and Michaelis-Menten plots using Origin, the kinetic parameters v_{max} and K_M are extracted and summed up in Table 3-1 for a first overview. In the following sub-chapters the results obtained from a number of measurements will be discussed in detail and depicted graphically to gain a deeper insight.

Table 3-1 Values of K_M and v_{max} for the kinetic characterization of the SADH-X2

substrate	c ($MgCl_2$) / mmol L^{-1}	MTBE	v_{max} / U mg^{-1}	K_M / mmol L^{-1}	K_S / mmol L^{-1}
2-butanone	-	-	3.8	98.4	-
2-butanone	1.0	-	10.0	380.4	-
2-butanone	-	saturated	1.7	173.0	-
acetone	-	-	3.5	1195.5	394.7
acetone	1.0	-	7.3	365.3	-
acetone	-	saturated	7.4	678.8	-
acetophenone	-	-	2.1	2.5	-
acetophenone	1.0	-	1.6	1.4	-
acetophenone	-	saturated	0.6	14.6	-
rac-2-butanol	-	-	1.1	71.9	-
rac-2-butanol	1.0	-	0.8	44.9	-
rac-2-butanol	-	saturated	0.6	91.4	-
2-propanol	-	-	3.6	316.4	-
2-propanol	1.0	-	3.3	330.0	-
2-propanol	-	saturated	0.4	29.2	-
rac-1-phenylethanol	-	-	0.7	1.3	-
rac-1-phenylethanol	1.0	-	0.6	1.6	-
rac-1-phenylethanol	-	saturated	0.1	4.8	-

3.3.2 Variation of Cofactor Concentration

The cofactor concentration of the nicotinamide cofactors NAD(P)H in their reduced and oxidized form is one of the key parameters when performing biocatalysis with enzymes dependent on one of these cofactors. Therefore, the activity of the SADH-X2 is investigated depending on the concentration of NADH and NAD^+ with 2-butanone and 2-propanol as substrates.

Depending on the NADH concentration a maximum of 0.7 U mg^{-1} at 0.3 mmol L^{-1} is observed (Figure 3-10). For the activity depending on the NAD^+ concentration in presence of 2-propanol a maximum of 0.6 U mg^{-1} at 0.8 mmol L^{-1} is observed (Figure 3-11). For further kinetic measurements it is important to choose a cofactor concentration for the standard assay where activity is not influenced by the cofactor concentration. With the multiplate reader it was not possible to obtain a linear dependency of the absorption on the cofactor concentration at concentrations close to and above 1.0 mmol L^{-1} NADH. Therefore, the concentration for further kinetic investigations is chosen to be 0.5 mmol L^{-1} which is in line with the results obtained by the industrial partner from the pre-screening.

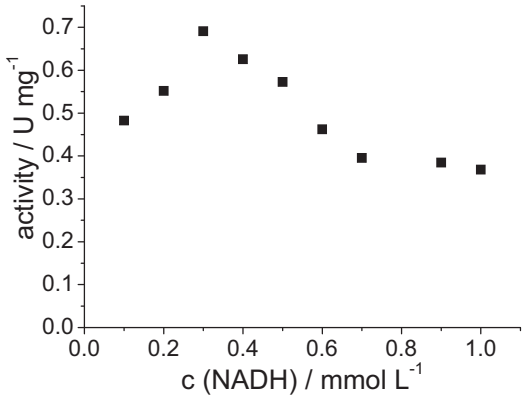


Figure 3-10 Variation of NADH concentration
(c (2-butanone) = 25 mmol L^{-1},
c (NADH) = 0.1-1.0 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

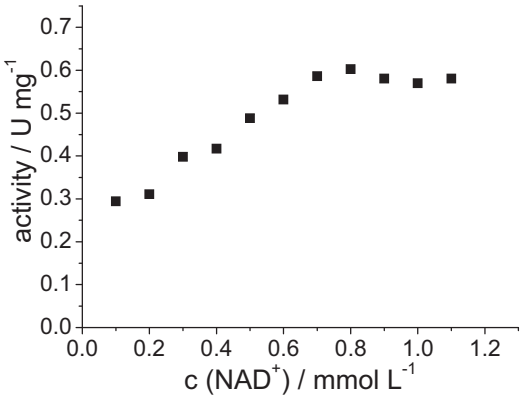


Figure 3-11 Variation of NAD^+ concentration
(c (2-propanol) = 25 mmol L^{-1},
c (NAD^+) = 0.1-1.1 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

3.3.3 Effect of Changes in Reaction Conditions in Presence of 2-Butanone

Depending on the 2-butanone concentration the activity of the SADH-X2 is first investigated in a 50 mmol L^{-1} buffer without any additives (Figure 3-12, Table 3-1). A typical Michaelis-Menten hyperbolic rate dependence is observed with v_{max} = 3.8 U mg^{-1} and a K_M value of 98.4 mmol L^{-1}. Comparing these results to the results obtained from the same measurements in presence of 1.0 mmol L^{-1} $MgCl_2$ (Figure 3-13, Table 3-1) shows that activity is higher if Mg^{2+} ions are present. The value of K_M is 380.4 mmol L^{-1}, v_{max} is 10.0 U mg^{-1}. Maximum reaction rate is 2.6-times higher in presence of $MgCl_2$. Mg^{2+} ions have an activating effect on the SADH-X2 which influences activity positively, but also leads to a higher K_M which implies a lower affinity of the ADH towards the substrate.

Since the ADH is to be used in one-phase systems as well as in two-phase systems, enzyme activity is measured in a 50 mmol L^{-1} buffer which is saturated with MTBE (Figure 3-14). In presence of the organic solvent v_{max} is 1.7 U mg^{-1} which is significantly lower than in both buffer systems without MTBE (Figure 3-12 and Figure 3-13).

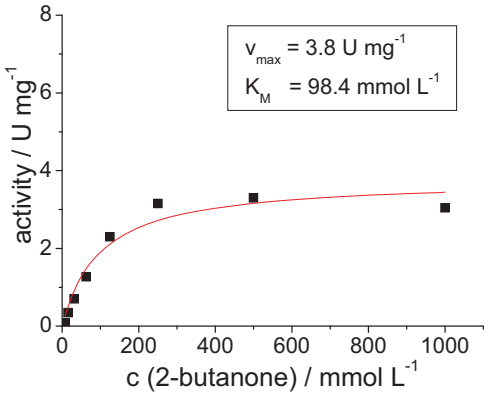


Figure 3-12 Variation of 2-butanone concentration
(c (substrate) = 7.8-1000 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

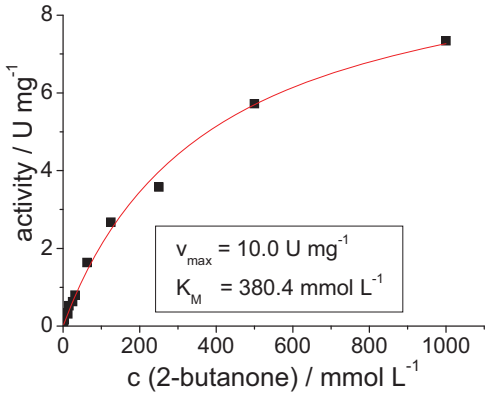


Figure 3-13 Effect of $MgCl_2$ addition in presence of 2-butanone
(c (substrate) = 1.6-1000 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

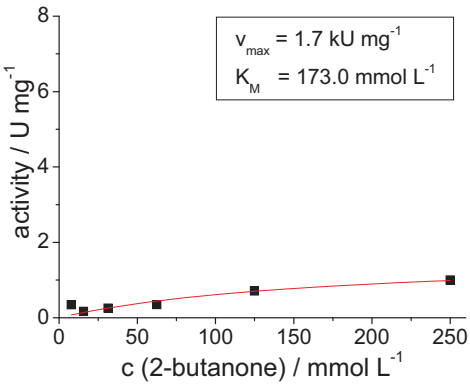


Figure 3-14 Effect of MTBE in presence of 2-butanone
(c (substrate) = 7.8-250 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.3.4 Effect of Changes in Reaction Conditions in Presence of Acetone

The activity for acetone is investigated because acetone is the co-product of the substrate dependent cofactor regeneration using 2-propanol. Therefore, it is important to know how active the enzyme is in presence of this co-product. In a 50 mmol L^{-1} buffer the SADH-X2 shows substrate inhibition (Figure 3-15, Table 3-1). With v_{max} = 3.5 U mg^{-1} maximum activity is higher than towards 2-butanone (Figure 3-12), but K_M of 1195.5 mmol L^{-1} reveals that the ADH has a higher affinity to 2-butanone than to acetone. Since these two substrates will both

be present in a reaction system where substrate dependent cofactor regeneration is applied this behaviour is beneficial for the progress of the reaction.

In presence of 1.0 mmol L^{-1} $MgCl_2$ v_{max} is 7.3 U mg^{-1} and K_M is 365.3 mmol L^{-1} (Figure 3-16, Table 3-1). The activating effect of Mg^{2+} ions is not as distinct as for 2-butanone (Figure 3-13).

In presence of MTBE the ADH shows a higher v_{max} and a higher K_M than in the system without MTBE (Figure 3-17, Table 3-1). With 7.4 U mg^{-1} v_{max} is even higher than in the presence of $MgCl_2$. The very high K_M of 678.8 mmol L^{-1} indicates that the enzyme loses its affinity towards acetone in presence of MTBE. This may be beneficial for the reaction system employing substrate dependent cofactor regeneration. In case of a low affinity of the ADH towards acetone this co-product will not inhibit the reduction of 2-butanone.

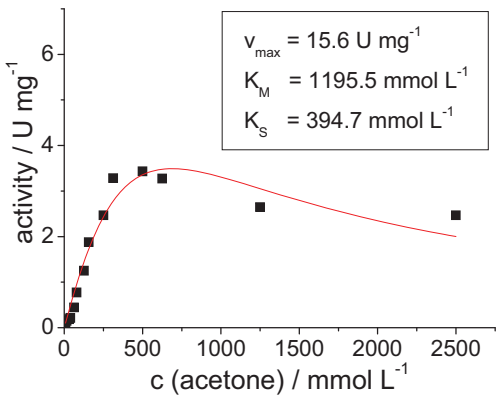


Figure 3-15 Variation of acetone concentration
(c (substrate) = 7.8-2500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

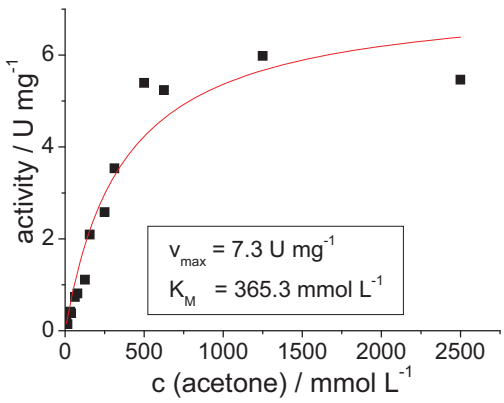


Figure 3-16 Effect of $MgCl_2$ in presence of acetone concentration
(c (substrate) = 7.8-2500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

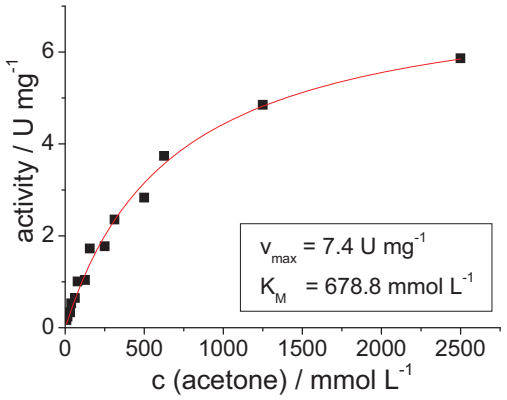


Figure 3-17 Effect of MTBE in presence of acetone
(c (substrate) = 7.8-2500 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (SADH-X2) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.3.5 Effect of Changes in Reaction Conditions in Presence of Acetophenone

The activity of the SADH-X2 towards acetophenone in a 50 mmol L^{-1} buffer is much lower than towards the non-aromatic ketones (Figure 3-18, Table 3-1). v_{max} is 2.1 U mg^{-1} which is about half as high as the values obtained for 2-butanone and acetone. The value for K_M is 2.5 mmol L^{-1} and indicates that the SADH-X2 has a high affinity towards acetophenone which is significantly higher than towards acetone. This is beneficial for the employment of substrate dependent cofactor regeneration because acetone will not compete strongly with acetophenone.

In presence of $MgCl_2$ v_{max} is 1.6 U mg^{-1} lower than in absence of the salt (Figure 3-19, Table 3-1). With 1.4 mmol L^{-1} K_M is only half of that observed in the absence of Mg^{2+} ions. In presence of the magnesium salt the ADH shows a higher affinity towards acetophenone than towards the non-aromatic ketones (Table 3-1). In contrast to the two tested alkyl ketones acetophenone has two very different R-groups, phenyl and methyl. Therefore, the orientation of the molecule in the active site of the enzyme is predefined. The active site of the SADH-X2 is known to be very small.[105] Hence, acetophenone cannot enter the active site with the phenyl group and is overall sterically more hindered which explains the low activity compared to the linear ketones.

In presence of MTBE the SADH-X2 shows a lower v_{max} of 0.6 U mg^{-1} and a higher K_M than in the absence of this solvent (Figure 3-20, Table 3-1). With 14.6 mmol L^{-1} K_M is higher than in the system containing no MTBE (Table 3-1). The affinity towards the aromatic ketone is

lower in the presence of MTBE but higher than for acetone at the same reaction conditions (Table 3-1).

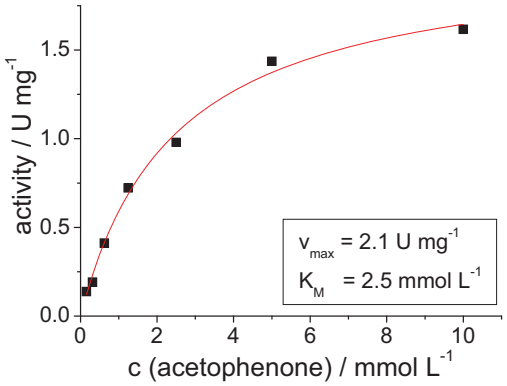


Figure 3-18 Variation of acetophenone concentration
(c (substrate) = 0.16-10.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

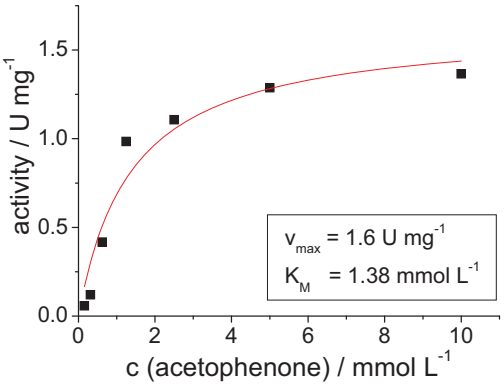


Figure 3-19 Effect of $MgCl_2$ in presence of acetophenone
(c (substrate) = 0.16-10.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mg mL^{-1})

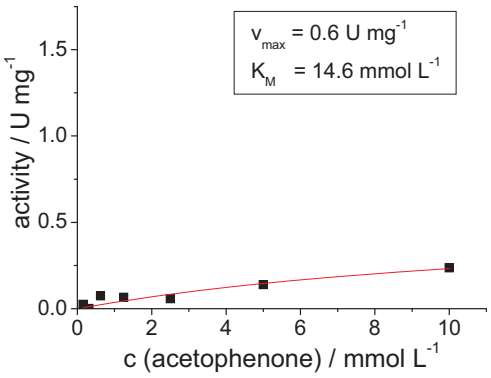


Figure 3-20 Effect of MTBE in presence of acetophenone concentration
(c (substrate) = 0.16-10.0 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.3.6 Effect of Changes in Reaction Conditions in Presence of *rac*-2-Butanol

The kinetic resolution of racemic alcohols by ADH catalysed oxidation is a means for the production of chiral alcohols. It may be interesting as the opposite enantiomer can be obtained with the same enzyme.[106] Oxidative cofactor regeneration can be carried out by

electrochemical means.[87] Furthermore, the characterization of the backward reaction allows quantification of product inhibition for the reduction.

In presence of *rac*-2-butanol in a 50 mmol L^{-1} buffer v_{max} is 1.1 U mg^{-1} (Figure 3-21). This value is small compared to the maximum activity obtained for the conversion of 2-butanone which is 3.8 U mg^{-1} (Table 3-1) under the same reaction conditions. Interestingly, K_M is 71.9 mmol L^{-1} for the racemic alcohol compared to 98.4 mmol L^{-1} for the ketone. The affinity of the SADH-X2 towards the racemic alcohol is 1.4-times higher than towards the substrate. The presence of 1.0 mmol L^{-1} $MgCl_2$ has a negative influence on v_{max} (Table 3-1). Due to the deactivation by Mg^{2+} ions v_{max} is decreased by 0.3 U mg^{-1} to 0.8 U mg^{-1}, and K_M is by a factor of 1.6 lower.

Saturation of the system with MTBE causes a significant decrease in v_{max} compared to the standard assay (Figure 3-22, Table 3-1). In contrast, K_M reaches 91.4 mmol L^{-1} which is about twice as high as in the system containing Mg^{2+} ions (Figure 3-23) and 1.3-times higher than in the system without $MgCl_2$ (Table 3-1). MTBE influences activity itself and increases K_M which means a lower affinity to the racemic product alcohol. This is beneficial for reaction rate and selectivity when performing the reduction of 2-butanone in the biphasic system buffer/MTBE.

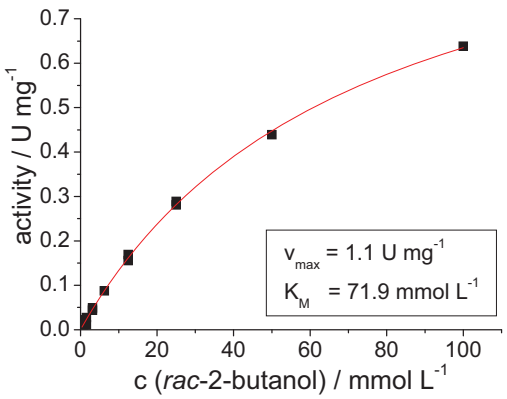


Figure 3-21 Variation of *rac*-2-butanol concentration
(c (substrate) = 0.39-100.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

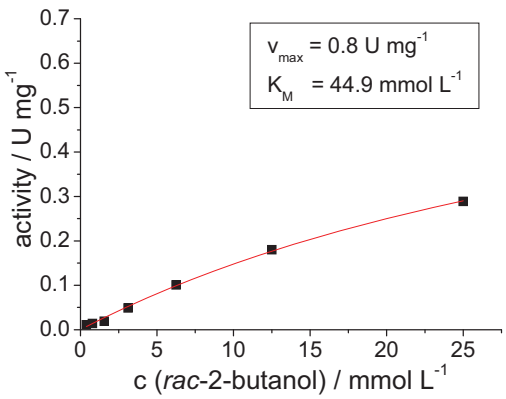


Figure 3-22 Effect of $MgCl_2$ in presence of *rac*-2-butanol
(c (substrate) = 0.39-25.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mg mL^{-1})

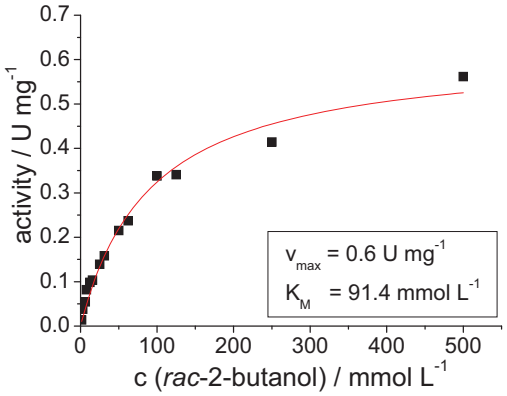


Figure 3-23 Effect of MTBE in presence of *rac*-2-butanol
(c (substrate) = 0.39-25.0 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (SADH-X2) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.3.7 Effect of Changes in Reaction Conditions in Presence of 2-Propanol

The non-chiral alcohol 2-propanol is used for substrate dependent cofactor regeneration. Therefore, activity towards this co-substrate is investigated as a function of concentration. In a 50 mmol L^{-1} buffer a typical Michaelis-Menten curve is obtained with v_{max} of 3.6 U mg^{-1} and K_M of 316.4 mmol L^{-1} (Figure 3-24). Maximum activity and K_M are in the same range as for acetone (Table 3-1). Comparing the results for *rac*-2-butanol and 2-propanol the affinity of the SADH-X2 towards the racemic alcohol is 4.4-times higher than towards the co-substrate. To prevent re-oxidation of 2-butanol in favor of the reduction of the oxidized cofactor a large excess of 2-propanol has to be used for the synthesis of the enantiopure alcohol.

The addition of 1.0 mmol L^{-1} $MgCl_2$ to the reaction mixture does not significantly influence v_{max} (Figure 3-25). Maximum activity is 0.3 U mg^{-1} lower than without $MgCl_2$, while K_M is decreased by 14 mmol L^{-1} which reveals a slightly higher affinity of the ADH in absence of Mg^{2+} ions. With 330.0 mmol L^{-1} K_M is 7.3-times higher than for *rac*-2-butanol (Table 3-1). In presence of MTBE the SADH-X2 loses its activity towards 2-propanol (Figure 3-26). v_{max} drops to 0.4 U mg^{-1} which is more than 9-times lower than without the solvent (Table 3-1). Due to the significant drop in maximum activity K_M also drops by a factor of about 10 to 29.2 mmol L^{-1}. The significant drop in maximum activity shows that a high excess of 2-propanol is needed to perform substrate dependent cofactor regeneration in a two-phase reaction system with MTBE as non-reactive phase although affinity is higher than towards *rac*-2-butanol.

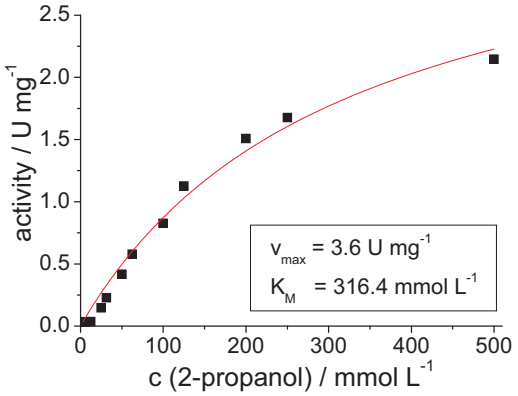


Figure 3-24 Variation of 2-propanol concentration
(c (substrate) = 7.8-500.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1})

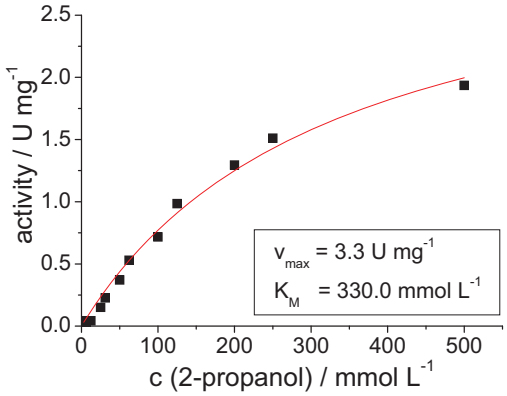


Figure 3-25 Effect of $MgCl_2$ in presence of 2-propanol
(c (substrate) = 3.1-500.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

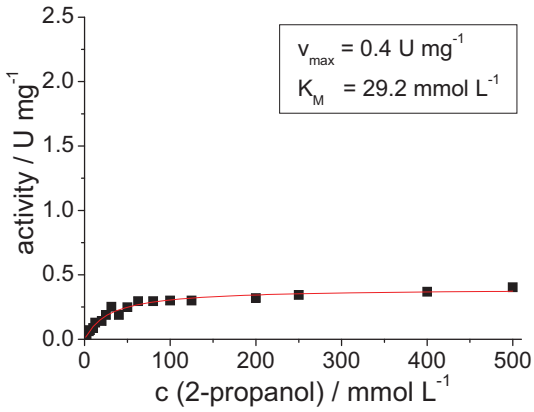


Figure 3-26 Effec of MTBE in presence of 2-propanol
(c (substrate) = 3.1-500.0 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (SADH-X2) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.3.8 Effect of Changes in Reaction Conditions in Presence of *rac*-1-Phenylethanol

Rac-1-phenylethanol is also applied as a substrate for kinetic resolution. In a 50 mmol L^{-1} buffer the ADH shows a maximum activity of 0.7 U mg^{-1} (Figure 3-27) which is by a factor of 3 lower than v_{max} towards the corresponding ketone acetophenone (Table 3-1). K_M reaches 1.3 mmol L^{-1} which shows that the SADH-X2 has a good affinity towards this alcohol. The value of K_M for acetophenone is 2-times higher (Table 3-1). This implies a risk of re-oxidation

of the product alcohol, but since the activity towards the ketone is 3-times higher than towards the racemic product alcohol the oxidation is not significantly contributing. Addition of 1.0 mmol L^{-1} $MgCl_2$ to the assay only causes slight increases in v_{max} to 0.6 U mg^{-1} and in K_M to 1.6 mmol L^{-1} (Figure 3-28). At the same reaction conditions v_{max} in presence of acetophenone is 2.7-times higher but affinity is in the same range (Table 3-1). In presence of MTBE v_{max} is decreased to 0.1 U mg^{-1} and K_M increases to 4.8 mmol L^{-1} (Figure 3-29). The presence of the additional solvent has a negative effect on the oxidation of the racemic mixture of 1-phenylethanol. At the same reaction conditions v_{max} in presence of acetophenone is six times higher, and the affinity is higher for the aromatic ketone. The obtained results do not reveal a risk of re-oxidation of 1-phenylethanol when performing the reduction of acetophenone which is in line with the finding that no racemisation occurs in batch experiments (Chapter 5.2.3).

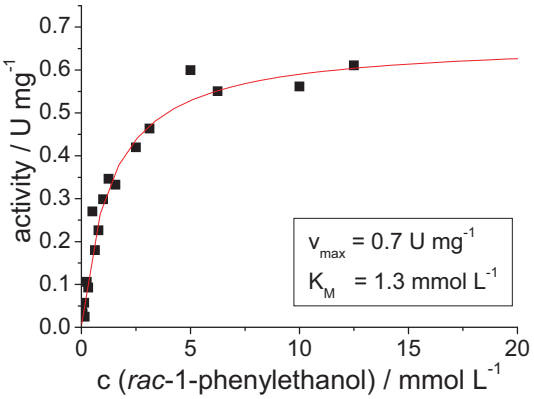


Figure 3-27 Variation of *rac*-1-phenylethanol concentration (c (substrate) = 0.02-20.0 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (SADH-X2) = 0.04 mg mL^{-1})

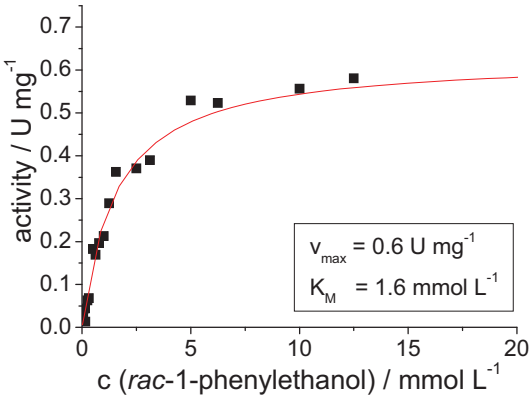


Figure 3-28 Effect of $MgCl_2$ in presence of *rac*-1-phenylethanol (c (substrate) = 0.02-20.0 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (SADH-X2) = 0.04 mg mL^{-1}, c ($MgCl_2$) = 1.0 mmol L^{-1})

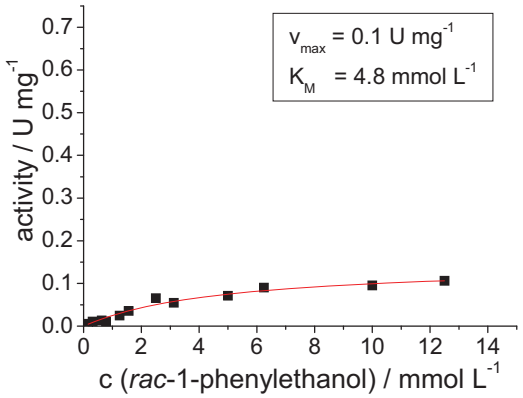


Figure 3-29 Effect of MTBE in presence of *rac*-1-phenylethanol
(c (substrate) = 0.02-12.25 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (SADH-X2) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.4 Kinetic Characterization of the LB-ADH

3.4.1 Introduction

The LB-ADH is the most investigated ADH because it is commercially available.[9, 30, 64, 80, 96, 107] Compared to other ADH it is exceptionally stable in presence of high concentrations of organic solvents. It exhibits a quaternary structure consisting of four monomers each containing one Mg^{2+} ion. The active site is formed by the four monomers and is located in the center. The LB-ADH is NADP-dependent and (*R*)-selective.

Two different preparations of the LB-ADH are kinetically characterized. A lyophilized preparation is compared to a liquid preparation which was prepared with an additional purification step. The dependence of activity on concentrations of substrate, cofactor, and buffer, and the effect of addition of $MgCl_2$ are investigated. Furthermore, the buffer which is used for the experiments is saturated with MTBE. This is done to test the activity of the LB-ADH in presence of high concentrations of organic solvents with regard to reactions in a two-phase reaction system.

3.4.2 Kinetic Characterization of the Lyophilized LB-ADH

From kinetic measurements for the characterization of the lyophilized LB-ADH and Michaelis-Menten plots the kinetic parameters v_{max} and K_M are extracted and summed up in Table 3-2 for a first overview. In the following sub-chapters the results obtained from a

number of measurements will be discussed in detail and depicted graphically to gain a deeper insight. Unfortunately, due to substrate excess inhibition it is not possible to fit all measurements to obtain kinetic parameters. When no K_M is given in the table v_{max} is only the maximum measured activity.

Table 3-2 Values of K_M and v_{max} for the kinetic characterization of the solid LB-ADH

substrate	c (buffer) / mmol L^{-1}	c ($MgCl_2$) / mmol L^{-1}	MTBE	v_{max} / U mg^{-1}	K_M / mmol L^{-1}	K_S / mmol L^{-1}
2-butanone	50.0	-	-	6.3	-	
2-butanone	500.0	-	-	3.2	-	
2-butanone	50.0	-	saturated	3.4	-	
2-butanone	50.0	1.0	-	6.3	0.1	
2-butanone	500.0	1.0	-	3.1	-	
acetone	50.0	-	-	4.6	-	
acetone	500.0	-	-	6.9	0.3	5409.0
acetone	50.0	-	saturated	3.3	0.1	19746.2
acetophenone	50.0	-	-	4.6	0.01	
acetophenone	500.0	-	-	6.1	0.5	
acetophenone	50.0	-	saturated	5.7	0.4	
acetophenone	50.0	1.0	-	9.7	0.7	
acetophenone	500.0	1.0	-	7.0	-	
rac-2-butanol	50.0	-	-	1.1	0.3	
rac-2-butanol	500.0	-	-	1.2	-	
rac-2-butanol	50.0	-	saturated	1.4	-	
2-propanol	50.0	-	-	0.7	1.1	
2-propanol	500.0	-	-	0.5	0.5	
2-propanol	50.0	-	saturated	0.5	4.4	
rac-1-phenylethanol	50.0	-	-	1.6	2.3	
rac-1-phenylethanol	500.0	-	-	1.1	1.2	
rac-1-phenylethanol	50.0	-	saturated	0.8	2.0	

3.4.2.1 Variation of Cofactor Concentration

The activity of the solid LB-ADH is first tested depending on the NADPH concentration. In presence of 25 mmol L^{-1} 2-butanone a v_{max} of 3.2 U mg^{-1} at 0.7 mmol L^{-1} $NADP^+$ is found (Figure 3-30). When acetone is used as substrate maximum activity is 4.1 U mg^{-1} at 0.5 mmol L^{-1} (Figure 3-31). Maximum activity in presence of acetone is higher than in presence of 2-butanone, but for acetone the maximum is less distinctive. Depending on the $NADP^+$ concentration the ADH shows a maximum in activity of 2.3 U mg^{-1} at 0.6 mmol L^{-1} in presence of *rac*-2-butanol (Figure 3-32). The increase in activity towards the maximum is only moderate as well as the decrease with further increasing $NADP^+$ concentration. In presence of 2-propanol no maximum is found (Figure 3-33). Obviously, the $NADP^+$

concentration does not have a significant influence on the LB-ADH activity. The obtained results show that depending on the $NADP^+$ concentration maximum activity towards *rac*-2-butanol is 3.1-times higher than in presence of 2-propanol. This result reveals that an excess of 2-propanol will be needed for the substrate dependent cofactor regeneration. When only small amounts of co-substrate are present the ADH will use the product alcohol for the regeneration.

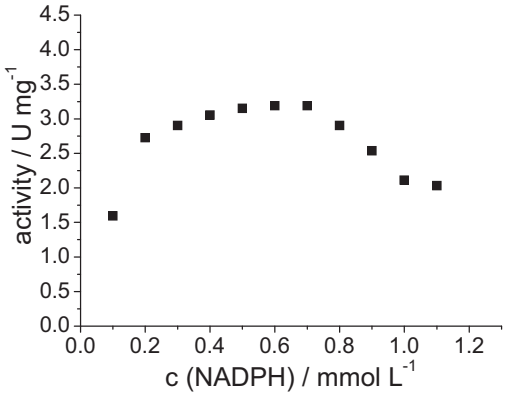


Figure 3-30 Variation of NADPH concentration in presence of 2-butanone (c (2-butanone) = 25 mmol L^{-1}, c (NADPH) = 0.1-1.1 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1})

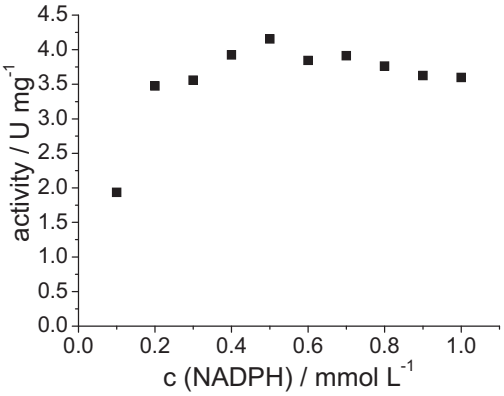


Figure 3-31 Variation of NADPH concentration in presence of acetone (c (acetone) = 25 mmol L^{-1}, c (NADPH) = 0.1-1.1 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1})

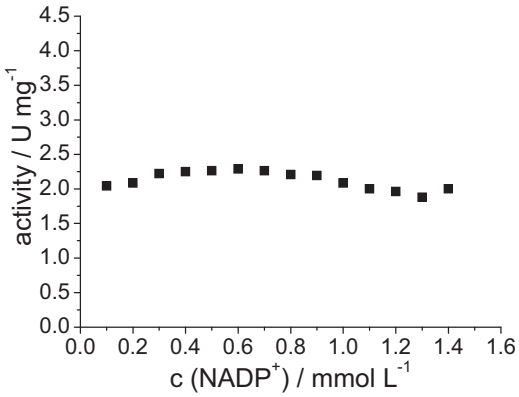


Figure 3-32 Variation of $NADP^+$ concentration in presence of *rac*-2-butanol (c (*rac*-2-butanol) = 25 mmol L^{-1}, c ($NADP^+$) = 0.1-1.6 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1})

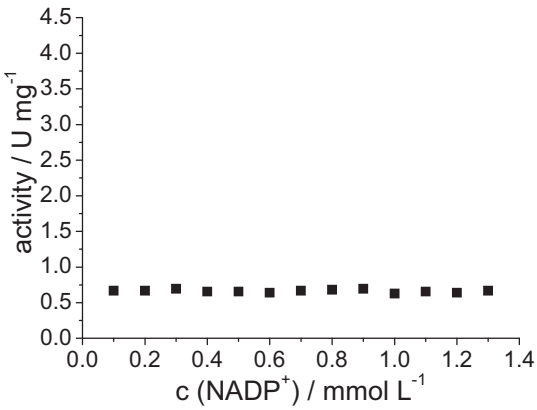


Figure 3-33 Variation of $NADP^+$ concentration in presence of *rac*-2-butanol (c (2-propanol) = 25 mmol L^{-1}, c ($NADP^+$) = 0.1-1.6 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1})

3.4.2.2 Effect of Changes in Reaction Conditions in Presence of 2-Butanone

In presence of 2-butanone in a 50 mmol L^{-1} buffer the LB-ADH shows substrate excess inhibition at concentrations higher than 1.0 mmol L^{-1} (Figure 3-34). Maximum measured reaction rate is 6.2 U mg^{-1} at 1.0 mmol L^{-1} 2-butanone. An increase in buffer concentration to 500 mmol L^{-1} causes a lower measured maximum rate of 3.2 U mg^{-1} reached at 0.63 mmol L^{-1} (Figure 3-35). Compared to the standard assay saturation of a 50 mmol L^{-1} buffer with MTBE also leads to a decrease in v_{max} to 3.4 U mg^{-1} at 7.81 mmol L^{-1} (Figure 3-36, Table 3-2). Substrate inhibition leads to a decrease in activity to 3.0 U mg^{-1} at 500 mmol L^{-1} 2-butanone. As for the SADH-X2 Mg^{2+} ions have an activating effect on the LB-ADH. Addition of 1.0 mmol L^{-1} $MgCl_2$ to the standard assay leads to a slight increase of v_{max} to 6.3 U mg^{-1} reached at 0.5 mmol L^{-1} (Figure 3-37). Again, substrate inhibition occurs and causes a decrease in activity to 2.3 U mg^{-1} at 500 mmol L^{-1} 2-butanone. In presence of Mg^{2+} ions the deactivating effect of the 500 mmol L^{-1} buffer is compensated (Figure 3-38). At a substrate concentration of 0.5 mmol L^{-1} a maximum rate of 6.1 U mg^{-1} is monitored which is twice as high as in the absence of $MgCl_2$ (Table 3-2).

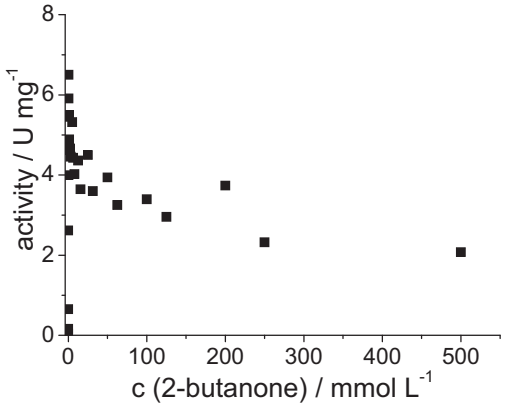


Figure 3-34 Variation of 2-butanone concentration
(c (substrate) = 0.02-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

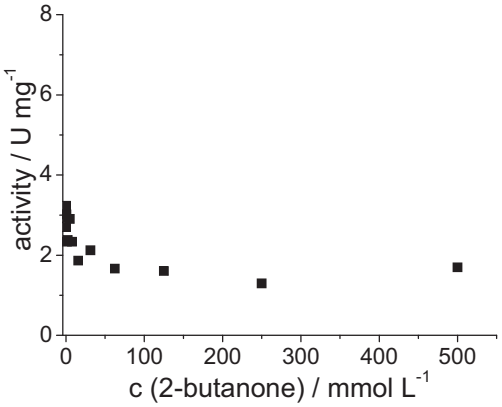


Figure 3-35 Effect of buffer concentration presence of 2-butanone
(c (substrate) = 0.02-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

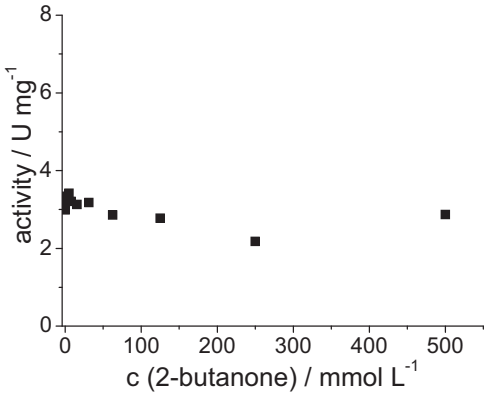


Figure 3-36 Effect of MTBE in presence of 2-butanone
(c (substrate) = 0.08-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

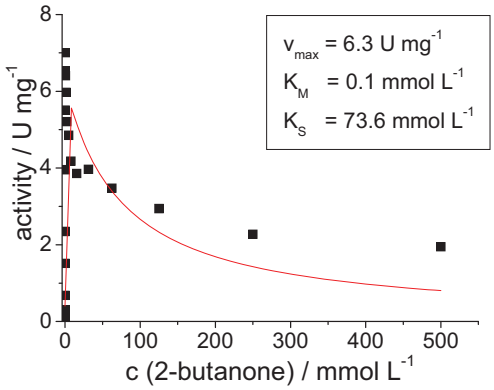


Figure 3-37 Effect of $MgCl_2$ in presence of 2-butanone
(c (substrate) = 0.02-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

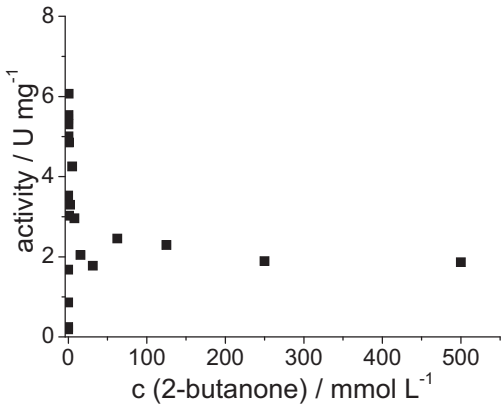


Figure 3-38 Effect of buffer concentration and $MgCl_2$ in presence of 2-butanone
(c (substrate) = 0.02-500 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1}, c ($MgCl_2$) = 1.0 mmol L^{-1})

3.4.2.3 Effect of Changes in Reaction Conditions in Presence of Acetone

The activity of the LB-ADH towards acetone is of interest because acetone is accepted as substrate and accumulates as a co-product of the substrate dependent cofactor regeneration when performing synthesis of enantiopure alcohols from prochiral ketones. In a 50 mmol L^{-1} buffer activity is hardly affected by the acetone concentration (Figure 3-39). At 5 mmol L^{-1} measured maximum activity of 4.6 U mg^{-1} is reached after only a slight increase in activity with increasing substrate concentration. Compared to 2-butanone activity is lower towards

acetone, but in presence of acetone substrate inhibition does not occur. A tenfold concentrated buffer activates the ADH in presence of acetone so that a v_{max} of 7.2 U mg^{-1} is reached (Figure 3-40). Saturation of the standard assay with MTBE causes the expected decrease in v_{max} to 3.6 U mg^{-1} (Figure 3-41). Acetone is a well-accepted substrate and may inhibit the enzyme when performing synthesis employing substrate dependent cofactor regeneration.

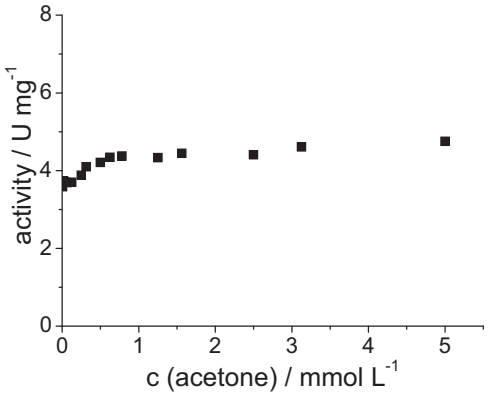


Figure 3-39 Variation of acetone concentration
(c (substrate) = 0.008-5.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

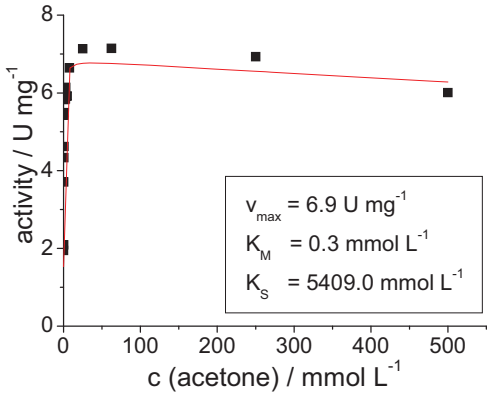


Figure 3-40 Effect of buffer concentration in presence of acetone
(c (substrate) = 0.008-500.0 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

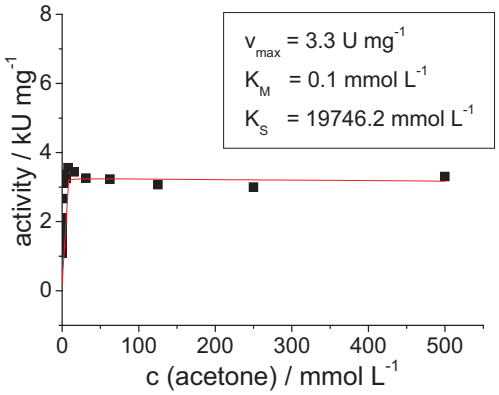


Figure 3-41 Effect of MTBE in presence of acetone
(c (substrate) = 0.008-500 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.4.2.4 Effect of Changes in Reaction Conditions in Presence of Acetophenone

With the standard assay a maximum in activity of 4.6 U mg^{-1} and a K_M of 0.01 mmol L^{-1} is observed (Figure 3-42). v_{max} is lower than towards 2-butanone (Table 3-2), and no substrate inhibition occurs. Increasing the buffer concentration to 500 mmol L^{-1} leads to an increase in v_{max} by a factor of 1.3 to 6.1 U mg^{-1} (Figure 3-43) while K_M is increased by a factor of 50 to 0.5 mmol L^{-1}. The high buffer concentration does not inhibit the LB-ADH as compared to measurements performed with 2-butanone. The same effect as for the high buffer concentration is observed when saturating the standard assay with MTBE (Figure 3-44). v_{max} increases to 5.7 U mg^{-1} while due to the 40-fold increase in K_M affinity of the ADH towards acetophenone is significantly decreased. The presence of 1.0 mmol L^{-1} $MgCl_2$ in the standard assay has a beneficial effect on v_{max} as well (Figure 3-45). Maximum activity is increased by a factor of 2.1 to 9.7 U mg^{-1} while K_M is increased to 0.7 mmol L^{-1} which implies a decrease in enzyme affinity by a factor of 70. Addition of 1.0 mmol L^{-1} $MgCl_2$ to a 500 mmol L^{-1} buffer does not affect K_M whereas v_{max} is increased to 7.0 U mg^{-1} (Figure 3-46) compared to the same measurements without salt addition (Table 3-2). As for the standard assay the presence of Mg^{2+} ions is beneficial for enzyme activity, but the system containing a 50 mmol L^{-1} buffer and $MgCl_2$ shows a higher K_M.

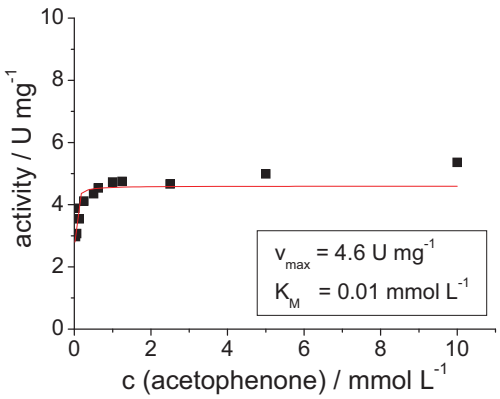


Figure 3-42 Variation of acetophenone concentration
(c (substrate) = 0.002-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

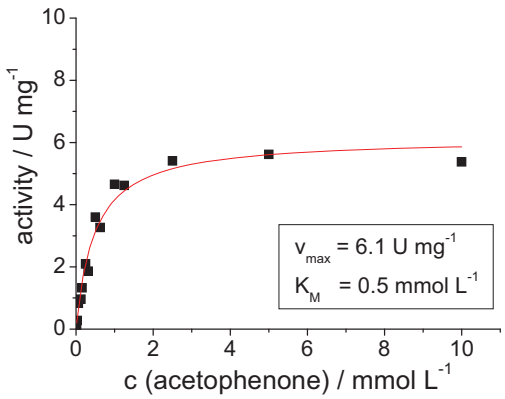


Figure 3-43 Effect of buffer concentration in presence of acetophenone
(c (substrate) = 0.02-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

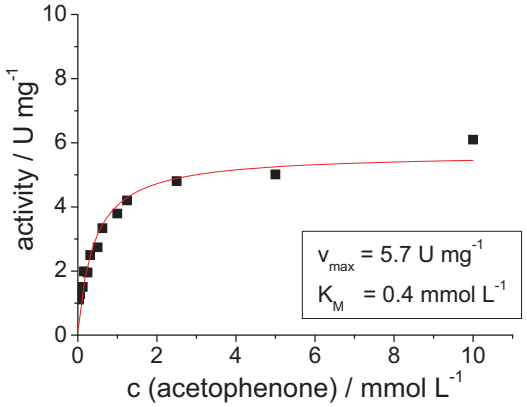


Figure 3-44 Effect of MTBE in presence of acetophenone
(c (substrate) = 0.02-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

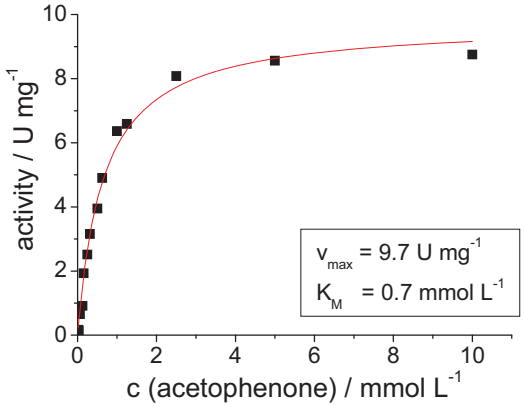


Figure 3-45 Effect of $MgCl_2$ in presence of acetophenone
(c (substrate) = 0.02-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

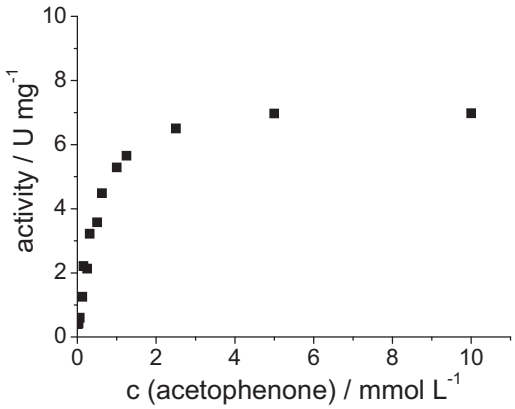


Figure 3-46 Effect of buffer concentration and $MgCl_2$ in presence of acetophenone
(c (substrate) = 0.02-10 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1}, c ($MgCl_2$) = 1.0 mmol L^{-1})

3.4.2.5 Effect of Changes in Reaction Conditions in Presence of *rac*-2-Butanol

ADH are known for their ability to catalyze both the reduction of a ketone and the oxidation of the corresponding alcohol. This implies a reversibility of the desired reduction of the ketone to the corresponding alcohol. Therefore, the activity of the LB-ADH depending on the *rac*-2-butanol concentration is tested. The activity-concentration plot for the standard assay shows a typical Michaelis-Menten dependence at low substrate concentrations. At

concentrations higher than 50 mmol L^{-1} a slight decrease in activity is observed (Figure 3-47) which hints towards substrate inhibition. v_{max} is 1.1 U mg^{-1} with a K_M of 0.3 mmol L^{-1}. Under the same reaction conditions maximum activity towards 2-butanone is higher (Table 3-2). Therefore, at low initial 2-butanone concentrations the ADH should not be inhibited by the product alcohol, but at high 2-butanol concentrations the re-oxidation may be favored. An increase in buffer concentration to 500 mmol L^{-1} leads to a slight increase of measured maximum rate to 1.2 U mg^{-1} (Figure 3-48). Again, a slight decrease in activity is observed after passing through the maximum. The deactivation of the high buffer concentration observed for 2-butanone does not occur for the corresponding alcohol. Compared to the standard assay the saturation with MTBE activates the LB-ADH and leads to an increase in maximum observed rate to 1.4 U mg^{-1} (Figure 3-49). At the same reaction conditions maximum activity towards 2-butanone is higher with 3.4 U mg^{-1} which should prevent preferred re-oxidation of the product alcohol.

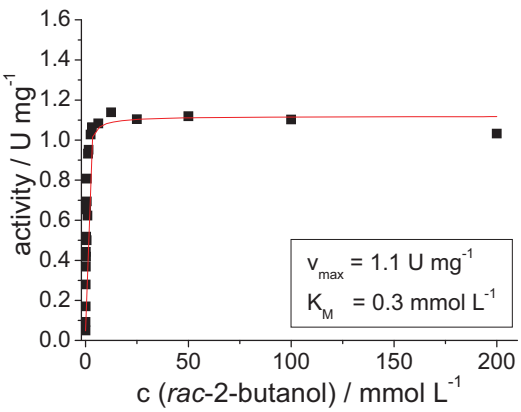


Figure 3-47 Variation of *rac*-2-butanol concentration
(c (substrate) = 0.02-200 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

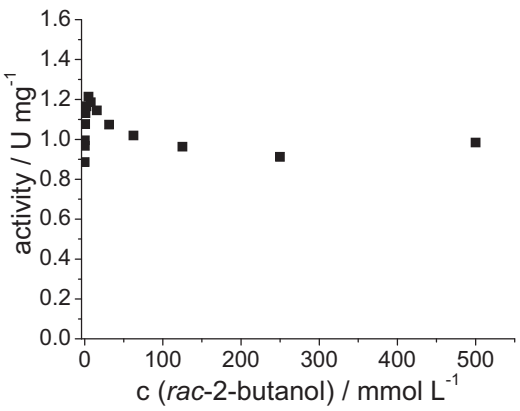


Figure 3-48 Effect of buffer concentration in presence of *rac*-2-butanol
(c (substrate) = 0.08-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

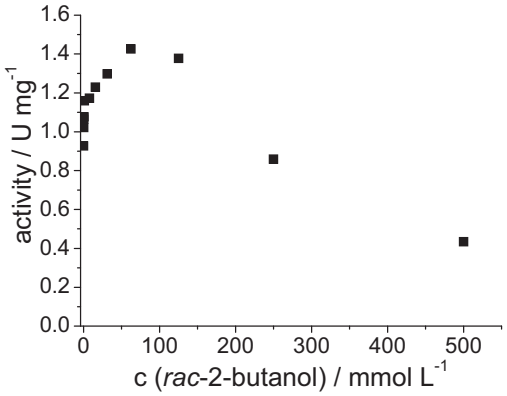


Figure 3-49 Effect of MTBE in presence of *rac*-2-butanol
(c (substrate) = 0.08-500 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.4.2.6 Effect of Changes in Reaction Conditions in Presence of 2-Propanol

The activity of the LB-ADH depending on the 2-propanol concentration is tested since 2-propanol will be used for substrate dependent cofactor regeneration. In a 50 mmol L^{-1} buffer the non-linear regression of the Michaelis-Menten plot indicates a v_{max} of 0.7 U mg^{-1} (Figure 3-50). Compared to the activity measurements with 2-butanone v_{max} is lower by factor of 9, but no substrate inhibition occurs. Therefore, for substrate dependent cofactor regeneration an excess of 2-propanol will be needed for sufficient rates of cofactor regeneration. Comparing the kinetic results for 2-propanol with those obtained for *rac*-2-butanol reveals that the re-oxidation of the product may be favored over the cofactor regeneration. In presence of 2-propanol a 500 mmol L^{-1} buffer has a deactivating effect on the LB-ADH (Figure 3-51). v_{max} is decreased to 0.5 U mg^{-1} which is lower than the maximum activity towards 2-butanone and *rac*-2-butanol (Table 3-2). This, again, indicates that for the cofactor regeneration a high excess of 2-propanol is needed. Saturation of the standard assay with MTBE leads to a decrease in activity (Figure 3-52, Table 3-2). Maximum activities in presence of 2-butanone and of the corresponding alcohol are higher at the same reaction conditions. Therefore, also in presence of MTBE an excess of 2-propanol is needed for synthesis.

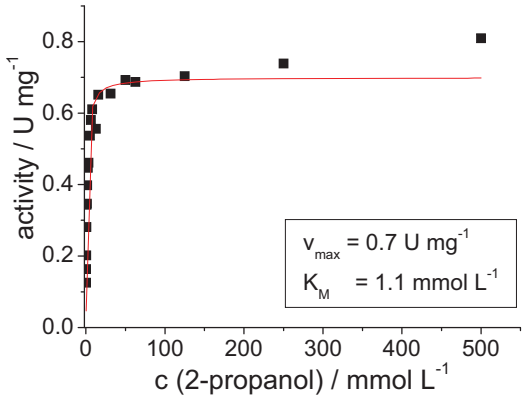


Figure 3-50 Variation of 2-propanol concentration
(c (substrate) = 0.08-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

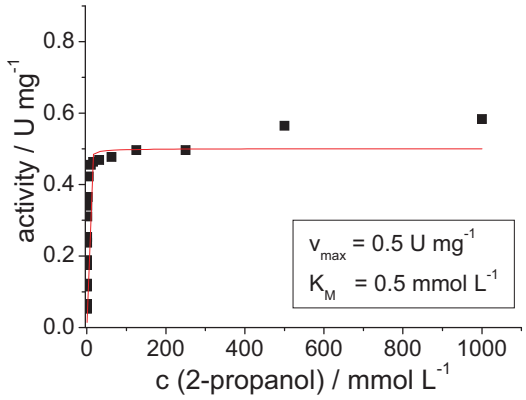


Figure 3-51 Effect of buffer concentration in presence of 2-propanol
(c (substrate) = 0.02-1000 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1})

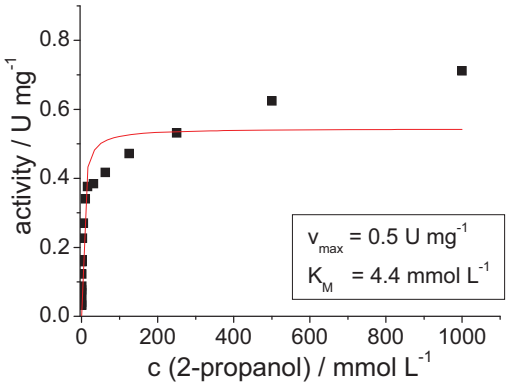


Figure 3-52 Effect of MTBE in presence of 2-propanol
(c (substrate) = 0.02-1000 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.4.2.7 Effect of Changes in Reaction Conditions in Presence of *rac*-1-Phenylethanol

For the model substrate acetophenone the corresponding alcohol is 1-phenylethanol which is also employed for kinetic investigations to learn about the possibility of kinetic resolution by enzyme catalysis. The standard assay results in a maximum activity of 1.6 U mg^{-1} and in a K_M value of 2.3 mmol L^{-1} (Figure 3-53). Activity towards the corresponding ketone is 2-times higher, and the enzyme has a 230-fold higher affinity towards the ketone (Table 3-2). For the competition with 2-propanol the same problem as for 2-butanol occurs. Activity towards the

co-substrate is lower than towards the product alcohol which makes an excess of 2-propanol necessary. An increase in buffer concentration to 500 mmol L^{-1} slightly deactivates the ADH (Figure 3-54). v_{max} is decreased to 1.1 U mg^{-1} while the affinity towards the racemic alcohol is increased by a factor of 2. Maximum activity towards acetophenone is higher. Therefore, re-oxidation should not take place. Comparing the values obtained for the aromatic alcohol with those measured for 2-propanol, again, the racemic alcohol would be the favored substrate. MTBE has an inhibitory effect on the LB-ADH in presence of 1-phenylethanol. v_{max} decreases by a factor of 2 to 0.8 U mg^{-1} (Figure 3-55). This kind of strong deactivation is not observed in the presence of acetophenone where the LB-ADH has a higher v_{max} and a higher affinity in the presence of MTBE (Table 3-2). Comparing the kinetic results obtained for the LB-ADH in presence of the aromatic alcohols with those measured in presence of the co-substrate 2-propanol (Table 3-2) shows that 1-phenylethanol will be the favored substrate for the cofactor regeneration if 2-propanol is not added with high excess.

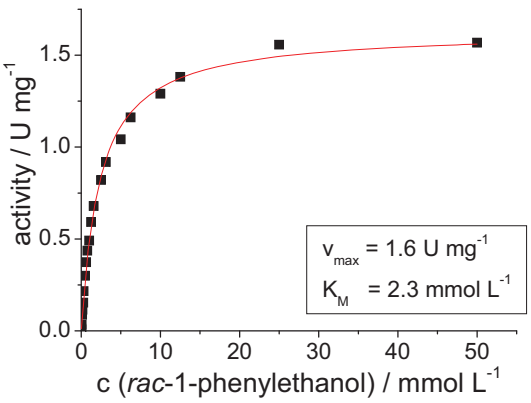


Figure 3-53 Variation of *rac*-1-phenylethanol concentration (c (substrate) = 0.02-50 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1})

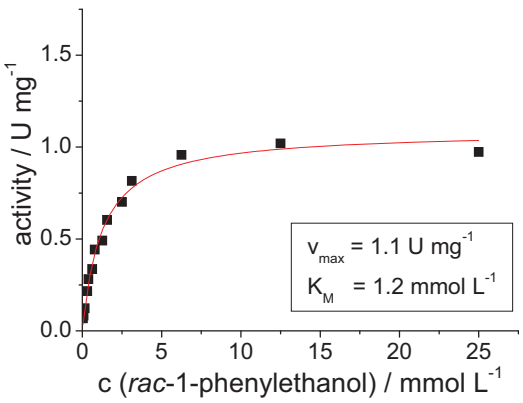


Figure 3-54 Effect of buffer concentration in presence of *rac*-1-phenylethanol (c (substrate) = 0.02-25 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 500 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1})

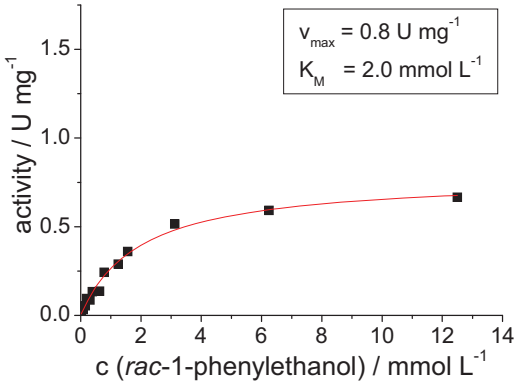


Figure 3-55 Effect of MTBE in presence of *rac*-1-phenylethanol
(c (substrate) = 0.02-12.6 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.04 mg mL^{-1}, buffer saturated with MTBE)

3.4.3 Kinetic Characterization of the Liquid LB-ADH

From kinetic measurements for the characterization of the liquid LB-ADH and Michaelis-Menten plots using Origin the kinetic parameters v_{max} and K_M are extracted and summed up in Table 3-3 for a first overview. In the following sub-chapters the results obtained from a number of measurements will be discussed in detail and depicted graphically to gain a deeper insight. Unfortunately, due to substrate inhibition it is not possible to fit all measurements to obtain kinetic parameters. When no K_M is given in the table v_{max} is only the maximum measured activity. A satisfying fit with an inhibition model cannot be obtained hinting towards a more complex interaction. As the target of the investigation is to test the influence of reaction conditions on activity, this is not further investigated.

Table 3-3 Values of K_M and v_{max} for the kinetic characterization of the liquid LB-ADH

substrate	c (buffer) / mmol L^{-1}	c ($MgCl_2$) / mmol L^{-1}	MTBE	v_{max} / U mL^{-1}	K_M / mmol L^{-1}
2-butanone	50.0	-	-	572.5	-
2-butanone	500.0	-	-	468.0	-
2-butanone	50.0	-	saturated	455.0	-
2-butanone	50.0	1.0	-	587.1	-
2-butanone	500.0	1.0	-	369.6	-
acetone	50.0	-	-	890.6	-
acetone	500.0	-	-	567.1	-
acetone	50.0	-	saturated	665.8	-
acetophenone	50.0	-	-	623.4	-
acetophenone	500.0	-	-	591.6	-
acetophenone	50.0	-	saturated	760.3	-
acetophenone	50.0	1.0	-	570.7	-
acetophenone	500.0	1.0	-	523.4	-
rac-2-butanol	50.0	-	-	79.4	-
rac-2-butanol	500.0	-	-	146.6	-
rac-2-butanol	50.0	-	saturated	168.4	-
2-propanol	50.0	-	-	31.6	1.5
2-propanol	500.0	-	-	46.6	-
2-propanol	50.0	-	saturated	32.5	1.8
rac-1-phenylethanol	50.0	-	-	47.9	-
rac-1-phenylethanol	500.0	-	-	73.9	-
rac-1-phenylethanol	50.0	-	saturated	69.0	1.7

3.4.3.1 Variation of Cofactor Concentration

In presence of 2-butanone, acetone, and of the corresponding alcohols the NADP(H) concentration is varied between 0.1 and 1.8 mmol L^{-1} to investigate the dependence of the liquid LB-ADH on the cofactor concentration. The plot of activity against concentration shows a maximum of 160.0 U mL^{-1} at 0.7 mmol L^{-1} NADPH in presence of 2-butanone (Figure 3-56). When the buffer is saturated with MTBE v_{max} of 201.7 U mL^{-1} is again found at 0.6 mmol L^{-1} and is in the same range as in the absence of MTBE (Figure 3-57).

In presence of acetone the ADH shows a slight increase in activity with increasing NADPH concentration to 169.0 U mL^{-1} (Figure 3-58). After having passed through the maximum activity decreases significantly with increasing NADPH concentration. Since acetone accumulates as co-product when synthesis of the enantiopure alcohols is performed it is important to enable a sufficient supply of reduced cofactor to suppress the reduction of acetone instead of 2-butanone. In presence of acetone saturation of the standard assay with MTBE has a larger effect on enzyme activity than in presence of 2-butanone (Figure 3-59). Maximum activity of 242.6 U mL^{-1} is reached at 0.5 mmol L^{-1}. As for 2-butanone maximum

activity is increased. The high ADH activity towards acetone enables its reduction instead of the substrate.

The activity-concentration plot for the dependence of the LB-ADH activity depending on the $NADP^+$ concentration in presence of *rac*-2-butanol shows a maximum activity of 85.4 U mL^{-1} at 0.2 mmol L^{-1} $NADP^+$ (Figure 3-60). In presence of 2-propanol maximum activity is 32.7 U mL^{-1} at 0.8 mmol L^{-1} (Figure 3-61), and is very low compared to *rac*-2-butanol. Therefore, a high excess of 2-propanol is necessary for the production of 2-butanol. For further kinetic characterization of the liquid LB-ADH a NADPH concentration of 0.5 mmol L^{-1} is chosen which is in line with the results obtained by the industry partner.

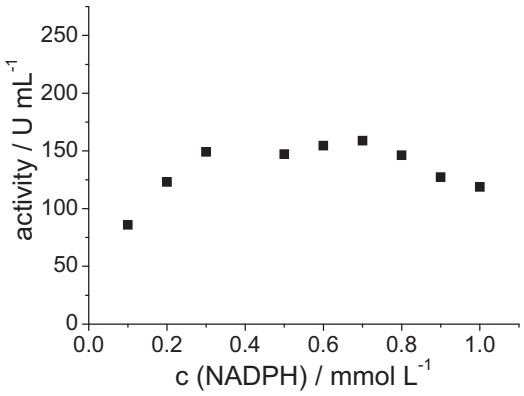


Figure 3-56 Variation of NADPH concentration in presence of 2-butanone
(c (2-butanone) = 25 mmol L^{-1},
c (NADPH) = 0.1-1.0 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

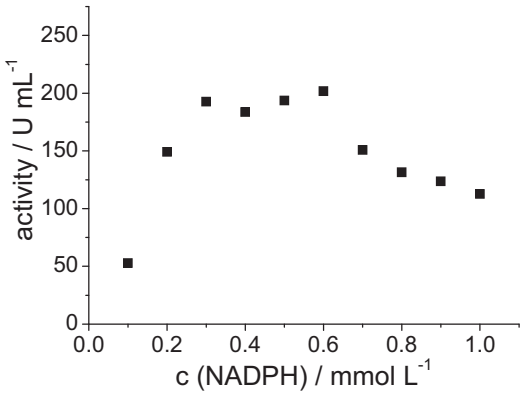


Figure 3-57 Variation of NADPH concentration in presence of 2-butanone and MTBE
(c (2-butanone) = 25 mmol L^{-1},
c (NADPH) = 0.1-1.0 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

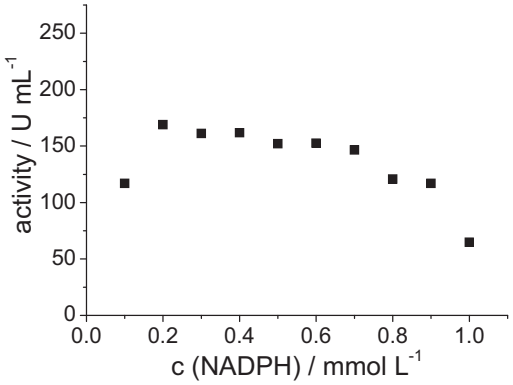


Figure 3-58 Variation of NADPH concentration in presence of acetone (c (acetone) = 25 mmol L^{-1}, c (NADPH) = 0.1-1.0 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1})

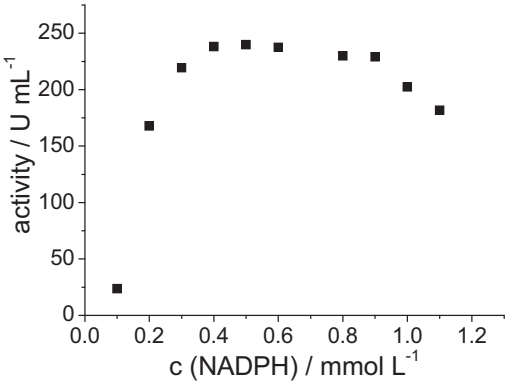


Figure 3-59 Variation of NADPH concentration in presence of acetone and MTBE (c (acetone) = 25 mmol L^{-1}, c (NADPH) = 0.1-1.0 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

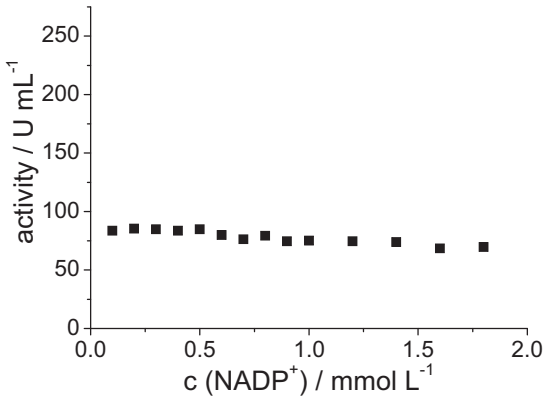


Figure 3-60 Variation of $NADP^+$ concentration in presence of *rac*-2-butanol (c (*rac*-2-butanol) = 25 mmol L^{-1}, c ($NADP^+$) = 0.1-1.0 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1})

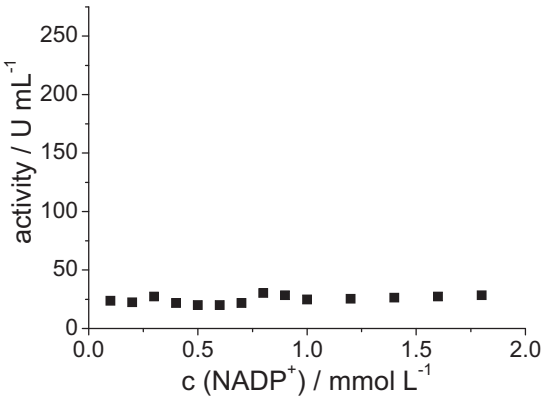


Figure 3-61 Variation of $NADP^+$ concentration in presence of 2-propanol (c (2-propanol) = 25 mmol L^{-1}, c ($NADP^+$) = 0.1-1.0 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1})

3.4.3.2 Effect of Changes in Reaction Conditions in Presence of 2-Butanone

In presence of a 50 mmol L^{-1} buffer a maximum activity of 675.0 U mL^{-1} at 0.5 mmol L^{-1} is observed (Figure 3-62). The activity-concentration plot reveals substrate inhibition which was already found for the solid LB-ADH preparation (see Chapter 3.3.3). A 10-fold increase in buffer concentration to 500 mmol L^{-1} leads to a 1.4-fold decrease in maximum rate to

475.0 U mL^{-1} due to enzyme deactivation (Figure 3-63). Furthermore, substrate excess inhibition is found. The presence of MTBE deactivates the ADH as well. Maximum observed rate is decreased by a factor of 1.5 to 460.0 U mL^{-1} (Figure 3-64), but product inhibition is less significant than for the standard assay. Compared to the corresponding measurements using the solid LB-ADH preparation (Table 3-2), the addition of 1.0 mmol L^{-1} $MgCl_2$ has a non-beneficial effect on the liquid LB-ADH (Figure 3-65, Table 3-3). Maximum activity is 580.0 U mL^{-1} and 1.2-fold lower than for the standard assay. Addition of $MgCl_2$ to a 500 mmol L^{-1} buffer cannot inhibit the deactivating effect of the high buffer concentration (Figure 3-66). Maximum measured activity is 475.0 U mL^{-1} which is the same value as in the pure 500 mmol L^{-1} buffer.

In view of the kinetic results discussed above it is the best choice to conduct the reduction of 2-butanone using the liquid LB-ADH in a 50 mmol L^{-1} buffer without any addition of $MgCl_2$. The presence of MTBE obviously decreases the maximum reachable activity. Furthermore, a two-phase system may have a beneficial effect due to extraction of the product alcohol and due to favorable equilibrium position.[108, 109]

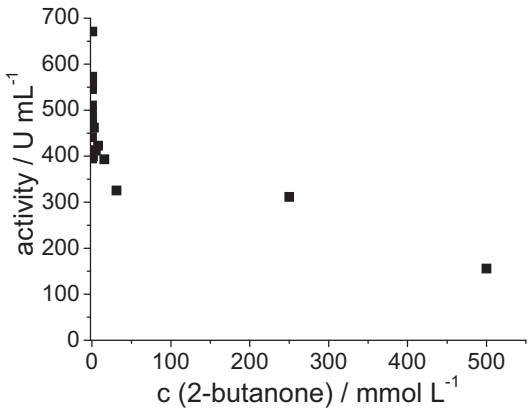


Figure 3-62 Variation of 2-butanone concentration
(c (substrate) = 0.008-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

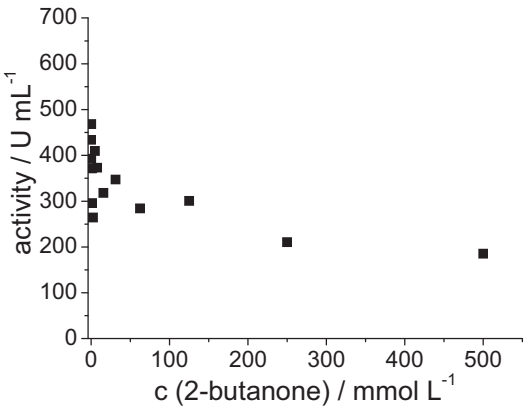


Figure 3-63 Effect of buffer concentration in presence of 2-butanone
(c (substrate) = 0.008-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

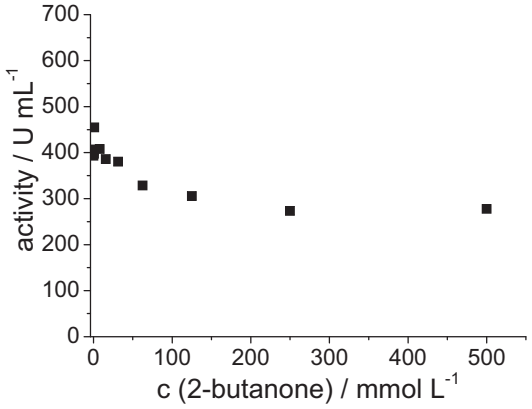


Figure 3-64 Effect of MTBE in presence of 2-butanone
(c (substrate) = 0.008-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

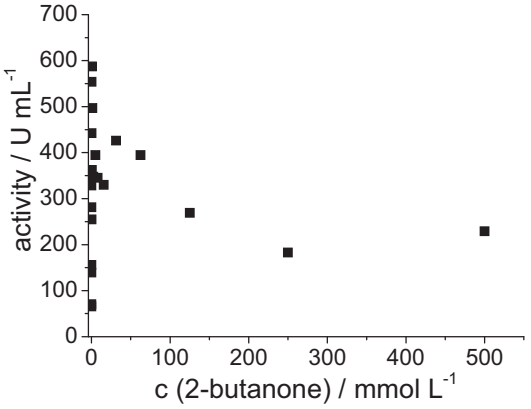


Figure 3-65 Effect of $MgCl_2$ in presence of 2-butanone
(c (substrate) = 0.008-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

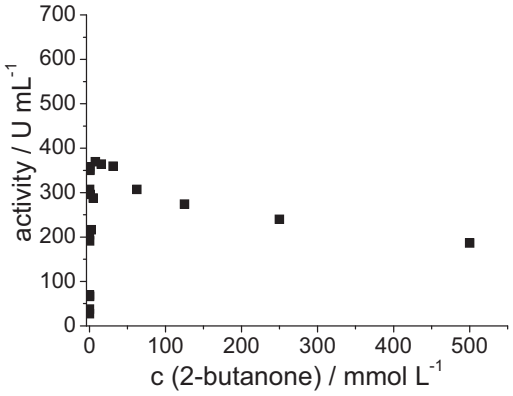


Figure 3-66 Effect of buffer concentration and MTBE in presence of 2-butanone
(c (substrate) = 0.008-500 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}, c ($MgCl_2$) = 1.0 mmol L^{-1})

3.4.3.3 Effect of Changes in Reaction Conditions in Presence of Acetone

Acetone is the co-product which is generated from the substrate dependent cofactor regeneration with 2-propanol. It accumulates in the synthesis of the chiral alcohols. Therefore, it is important to investigate the activity of the LB-ADH towards this co-product since it might compete with the substrate.

In a 50 mmol L^{-1} buffer apparent maximum activity is 850.0 U mL^{-1} (Figure 3-67). In contrast to the solid LB-ADH preparation, substrate inhibition is observed. Since at the same reaction conditions activity towards 2-butanone is lower (Table 3-3) a competition between acetone and 2-butanone might be possible. An increase in buffer concentration to 500 mmol L^{-1} causes a decrease in measured maximum activity to 560.0 U mL^{-1} (Figure 3-68) due to enzyme deactivation by the high buffer concentration. Compared to the standard assay substrate inhibition is less significant. Saturation of the standard assay with MTBE deactivates the LB-ADH (Figure 3-69). Maximum reaction rate is decreased to 560.0 U mL^{-1}, but substrate inhibition does not occur. For all three systems measured maximum activity for acetone is higher than towards 2-butanone under the same conditions. This might lead to inhibition of the enzyme by the co-product when performing the reduction of 2-butanone using substrate dependent cofactor regeneration.

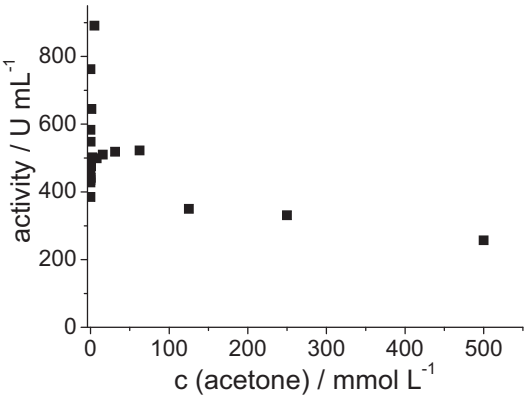


Figure 3-67 Variation of acetone concentration
(c (substrate) = 0.008-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

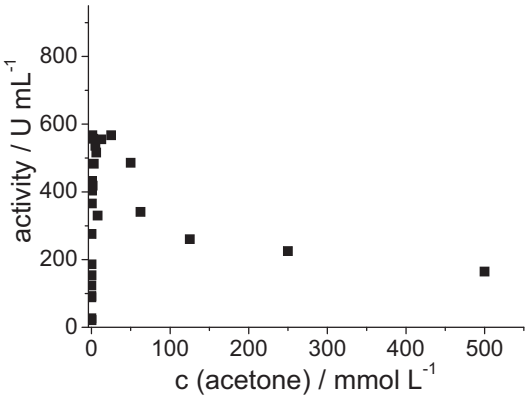


Figure 3-68 Effect of buffer concentration in presence of acetone
(c (substrate) = 0.008-500 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

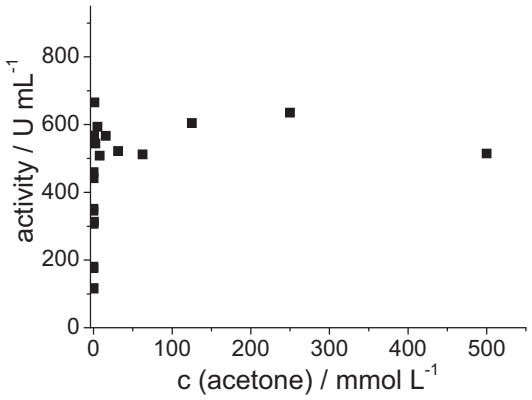


Figure 3-69 Effect of MTBE in presence of acetone
(c (substrate) = 0.02-500 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

3.4.3.4 Effect of Changes in Reaction Conditions in Presence of Acetophenone

The activity of the liquid LB-ADH preparation is determined depending on the concentration of the model substrate acetophenone. In presence of a 50 mmol L^{-1} buffer maximum activity is 620.0 U mL^{-1} (Figure 3-70). Substrate inhibition is not observed, but a non-linear regression using Michaelis-Menten kinetics is not possible. Compared to v_{max} of acetone at the same reaction conditions (Table 3-3) acetone is the favored substrate which might lead to the mentioned inhibition of the LB-ADH. An increase in buffer concentration to 500 mmol L^{-1} does not cause the expected deactivation of the enzyme but leads to a slight increase in activity by a factor of 1.1 to 660.0 U mL^{-1} (Figure 3-71). At these reaction conditions activity towards acetophenone is slightly higher than towards acetone (Table 3-3). The presence of MTBE in the standard assay activates the LB-ADH. Measured maximum activity is increased to 760.0 U mL^{-1} (Figure 3-72). Compared to acetone this value is 1.2-times higher, and, therefore, inhibition of the ADH by the co-product should not occur. The addition of 1.0 mmol L^{-1} $MgCl_2$ influences enzyme activity (Figure 3-73). Maximum activity is 550.0 U mL^{-1} which is a decrease of 70.0 U mL^{-1}. In a 500 mmol L^{-1} buffer the presence of $MgCl_2$ causes a decrease in activity by a factor of 1.3 to 520.0 U mL^{-1} compared to the system without $MgCl_2$ (Figure 3-74, Table 3-3). The results show that it is beneficial to use $MgCl_2$ in the presence of a 50 mmol L^{-1} buffer, but not at a buffer concentration of 500 mmol L^{-1}.

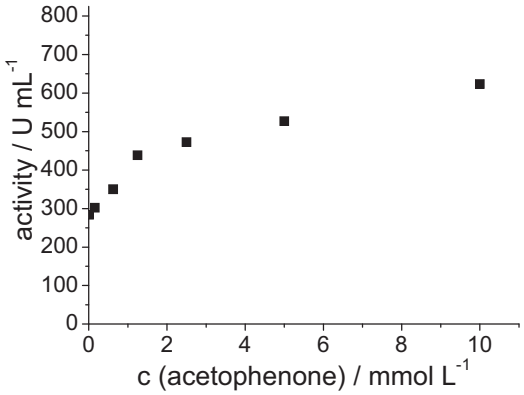


Figure 3-70 Variation of acetophenone concentration
(c (substrate) = 0.004-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

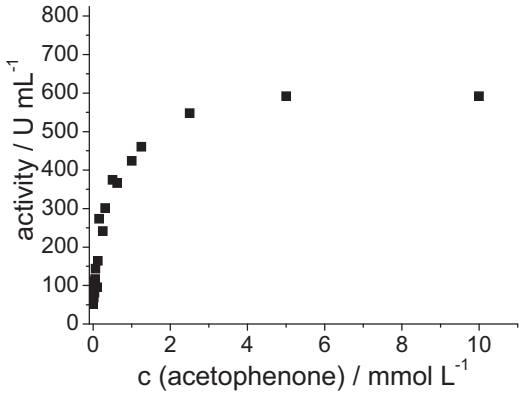


Figure 3-71 Effect of buffer concentration in presence of acetophenone
(c (substrate) = 0.004-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

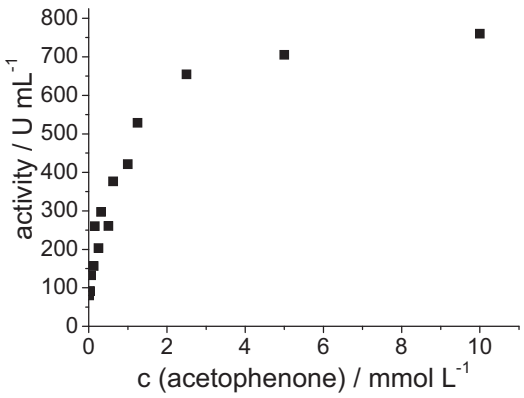


Figure 3-72 Effect of MTBE in presence of acetophenone
(c (substrate) = 0.004-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

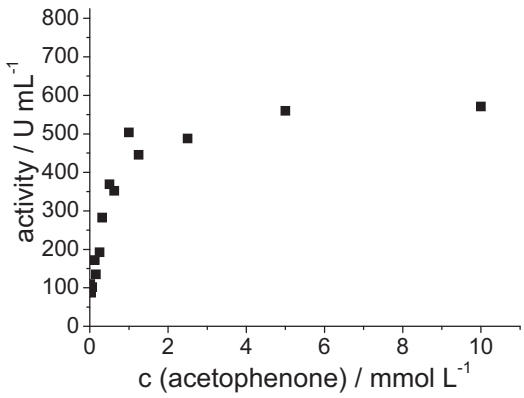


Figure 3-73 Effect of $MgCl_2$ in presence of acetophenone
(c (substrate) = 0.004-10 mmol L^{-1},
c (NADPH) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1},
c ($MgCl_2$) = 1.0 mmol L^{-1})

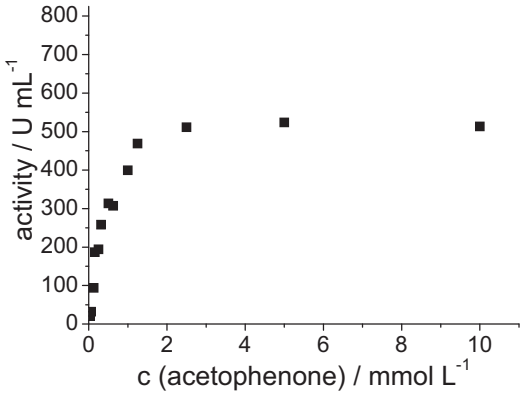


Figure 3-74 Effect of buffer concentration and $MgCl_2$ in presence of acetophenone (c (substrate) = 0.004-10 mmol L^{-1}, c (NADPH) = 0.5 mmol L^{-1}, c (buffer) = 500 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1}, c ($MgCl_2$) = 1.0 mmol L^{-1})

3.4.3.5 Effect of Changes in Reaction Conditions in Presence of *rac*-2-Butanol

Since ADH do not only catalyze the reduction of ketones but the oxidation of alcohols as well, activity of the liquid LB-ADH is investigated depending on the *rac*-2-butanol concentration. Maximum measured activity obtained in a 50 mmol L^{-1} buffer is 78.0 U mL^{-1} (Figure 3-75) which is 8.7-times lower than in presence of 2-butanone. An increase in buffer concentration to 500 mmol L^{-1} activates the ADH (Figure 3-76). v_{max} is increased by a factor of 2.1 to 160.0 U mL^{-1}. Substrate inhibition is observed, but at the same reaction conditions activity towards 2-butanone is 3-times higher. Saturation with MTBE increases v_{max} to 170.0 U mL^{-1} which shows a clear activating effect on the ADH (Table 3-3). Again, maximum activity towards 2-butanone is higher by a factor of 2.7. Although for all three investigated systems maximum activity is higher towards 2-butanone than towards the racemic product alcohol re-oxidation of the product alcohol may occur if not sufficient amounts of 2-propanol are available for cofactor regeneration. In presence of 2-propanol maximum activities for all three assays (Figure 3-50 to Figure 3-52) are lower compared to *rac*-2-butanol. A change in kinetic conditions due to increasing product concentration and decreasing substrate concentration may improve the conditions for product oxidation. Therefore, high concentrations of the product alcohol have to be avoided. This can be realized in a two-phase reaction system by in situ extraction of the alcohol out of the reactive phase.

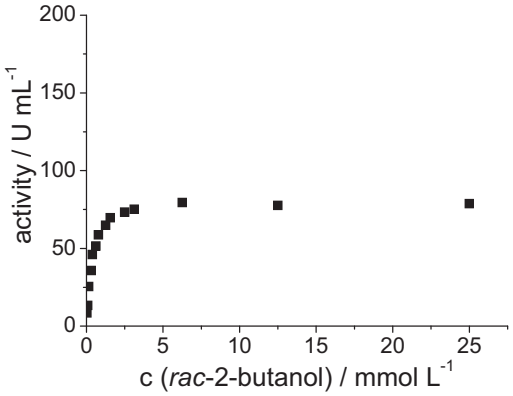


Figure 3-75 Variation of *rac*-2-butanol concentration
(c (substrate) = 0.004-10 mmol L^{-1},
c ($NADP^+$) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

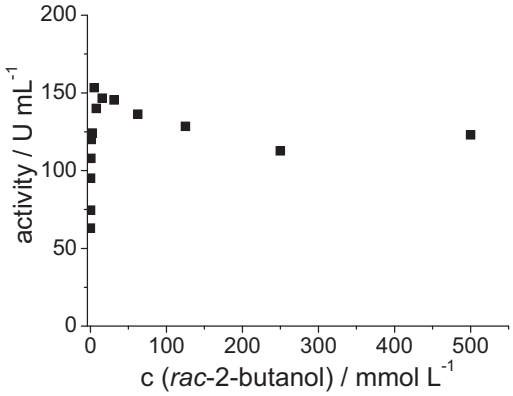


Figure 3-76 Effect of buffer concentration in presence of *rac*-2-butanol
(c (substrate) = 0.004-10 mmol L^{-1},
c ($NADP^+$) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

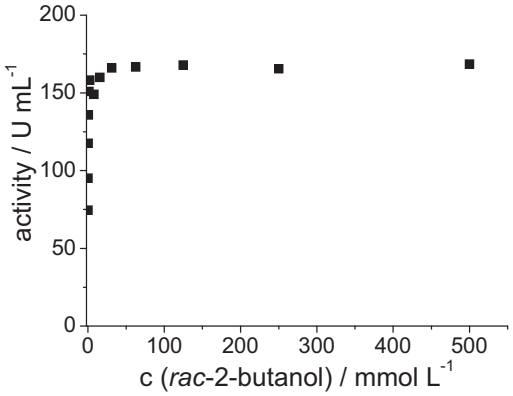


Figure 3-77 Effect of MTBE in presence of *rac*-2-butanol concentration
(c (substrate) = 0.004-10 mmol L^{-1}, c ($NADP^+$) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

3.4.3.6 Effect of Changes in Reaction Conditions in Presence of 2-Propanol

For the substrate dependent cofactor regeneration 2-propanol is needed as co-substrate. Therefore, the ADH activity towards this non-chiral alcohol is investigated. In a 50 mmol L^{-1} buffer v_{max} of 31.6 U mL^{-1} is found (Figure 3-78) which is 2.5-times lower than towards *rac*-2-butanol (Table 3-3). Although a 10-fold increase in buffer concentration increases maximum reaction rate by a factor of 1.5 to 46.0 U mL^{-1} (Figure 3-79) this value is still by a factor of 3.5 lower than in presence of *rac*-2-butanol. Saturation with MTBE does not affect

v_{max} (Figure 3-80). The K_M values are low for the standard assay and for the MTBE containing assay (Table 3-3) and promise good affinity of the ADH towards 2-propanol. The maximum activities show that an excess of 2-propanol will be essential for the synthesis of chiral alcohols when employing substrate dependent cofactor regeneration, especially for the synthesis of 2-butanol.

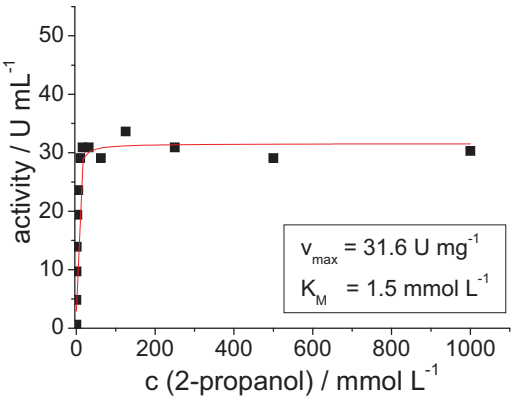


Figure 3-78 Variation of 2-propanol concentration
(c (substrate) = 0.02-1000 mmol L^{-1},
c ($NADP^+$) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1})

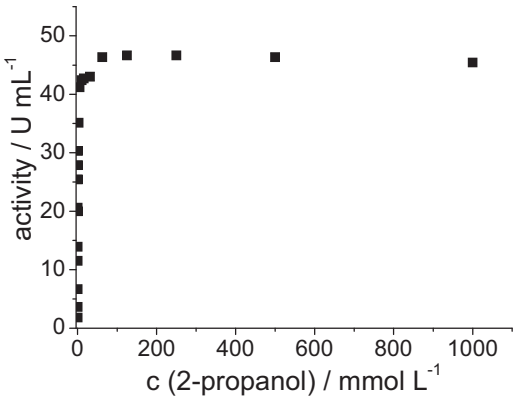


Figure 3-79 Effect of buffer concentration in presence of 2-propanol
(c (substrate) = 0.02-1000 mmol L^{-1},
c ($NADP^+$) = 0.5 mmol L^{-1},
c (buffer) = 500 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}

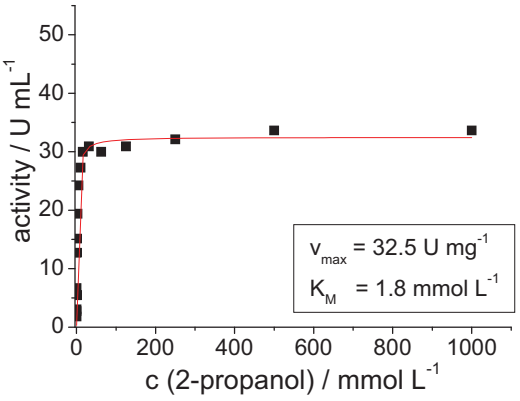


Figure 3-80 Effect of MTBE in presence of 2-propanol concentration
(c (substrate) = 0.02-1000 mmol L^{-1}, c ($NADP^+$) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1},
c (LB-ADH) = 0.9 μL mL^{-1}, buffer saturated with MTBE)

3.4.3.7 Effect of Changes in Reaction Conditions in Presence of *rac*-1-Phenylethanol

In a 50 mmol L^{-1} buffer maximum measured activity is 48.0 U mL^{-1} (Figure 3-81) which is 13-times lower than the maximum activity obtained in presence of the corresponding ketone acetophenone (Table 3-3). In contrast, maximum activity towards 2-propanol is 1.5-times lower (Table 3-3). An increase in buffer concentration to 500 mmol L^{-1} activates the ADH (Figure 3-82). Maximum activity is increased by 1.5 to 72.0 U mL^{-1} which is half as high as v_{max} obtained in presence of 2-propanol (Table 3-3). Saturation of the standard assay with MTBE has a beneficial effect on v_{max}. It increases by a factor of 1.4 to 69.0 U mL^{-1} (Figure 3-83). Here, maximum activity towards 2-propanol is only half as high (Table 3-3). As already mentioned for the reaction system containing 2-butanol, the obtained results reveal the necessity of an excess of 2-propanol to avoid re-oxidation of the aromatic product alcohol in the course of the cofactor regeneration.

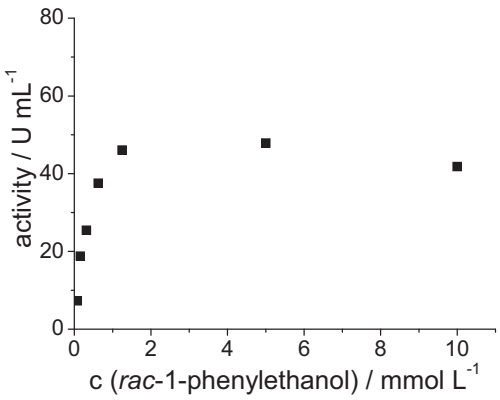


Figure 3-81 Variation of *rac*-1-phenylethanol concentration (c (substrate) = 0.02-10 mmol L^{-1}, c ($NADP^+$) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1})

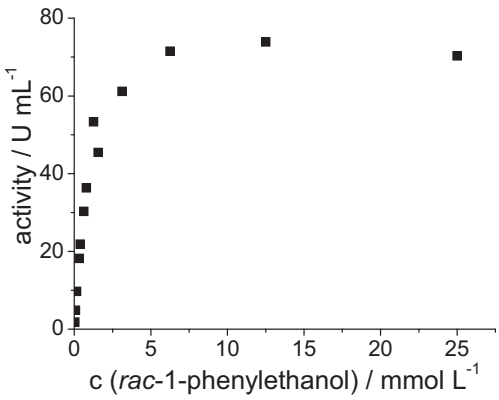


Figure 3-82 Effect of buffer concentration in presence of *rac*-1-phenylethanol (c (substrate) = 0.02-10 mmol L^{-1}, c ($NADP^+$) = 0.5 mmol L^{-1}, c (buffer) = 500 mmol L^{-1}, c (LB-ADH) = 0.9 μL mL^{-1})

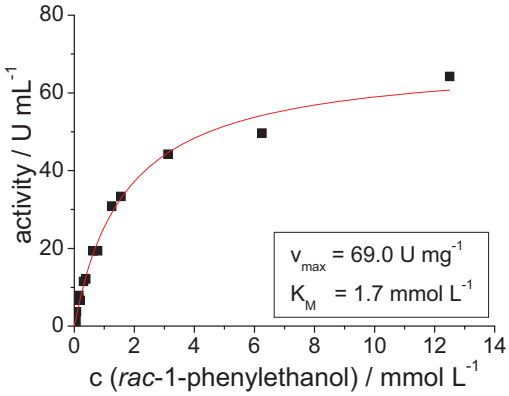
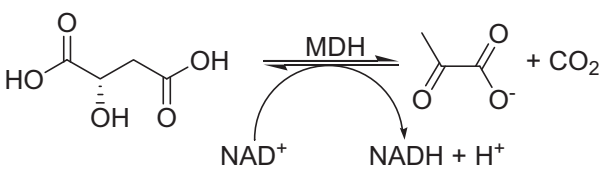


Figure 3-83 Effect of MTBE in presence of *rac*-1-phenylethanol concentration (c (substrate) = 0.02-10 mmol L^{-1}, c ($NADP^+$) = 0.5 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (LB-ADH) = 0.9 µL mL^{-1}, buffer saturated with MTBE)

3.5 Kinetic Characterization of the NAD-dependent MDH

The NAD-dependent malate dehydrogenase (MDH) is evaluated for cofactor regeneration. It oxidizes *L*-malic acid to pyruvate and CO_2 under condommittant reduction of NAD^+ to NADH (Scheme 3-2). The activity of the MDH is investigated depending on:

- concentration of *L*-malic acid
- concentration of NAD^+
- presence and concentration of $MnCl_2$
- incubation of MDH and NAD^+ with $MnCl_2$
- pH value



Scheme 3-2 Reduction of NAD^+ using the NAD-dependent MDH

Depending on the *L*-malic acid concentration the MDH has a maximum in activity of 0.034 U mg^{-1} which is reached at 100.0 mmol L^{-1} (Figure 3-84) whereas the activity values are scattered at concentrations lower than 200 mmol L^{-1}. Substrate inhibition is observed converging towards an equilibrium activity of about 0.02 U mg^{-1}. The addition of $MnCl_2$

increases v_{max} significantly by a factor of 44 to 1.5 U mg^{-1} (Figure 3-85). Substrate inhibition is not observed, and K_M of 38.3 U mg^{-1} shows a good affinity of the MDH towards *L*-malic acid. The pH value is known to have an influence on enzyme activity. Therefore, it is increased from 6.0 to 6.5. This leads to an increase in activity by a factor of 1.2 to 0.04 U mg^{-1} (Figure 3-86). Addition of 1.0 mmol L^{-1} $MnCl_2$ enables a further increase in activity to 2.4 U mg^{-1} which is 1.6-times higher than in the presence of Mn^{2+} ions at pH 6.0 (Figure 3-87).

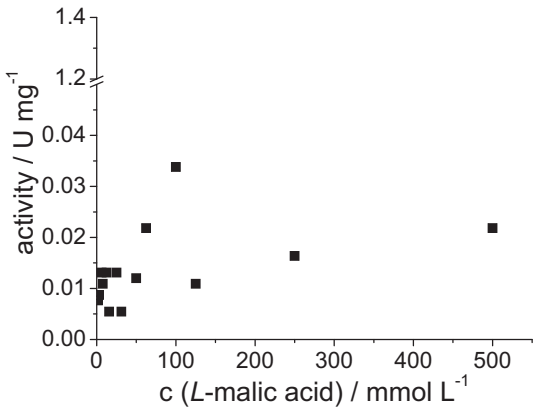


Figure 3-84 Variation of *L*-malic acid concentration
(c (substrate) = 1.56-500 mmol L^{-1},
c (NAD^+) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

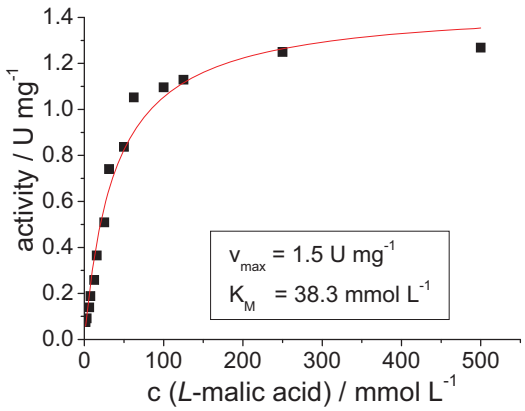


Figure 3-85 Effect of $MnCl_2$ in presence of *L*-malic acid
(c (substrate) = 1.56-500 mmol L^{-1},
c (NAD^+) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1},
c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.0)

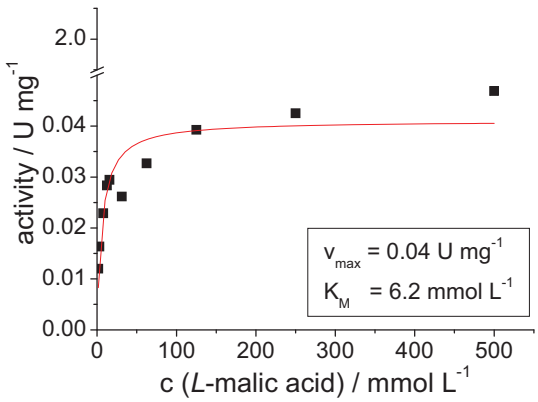


Figure 3-86 Effect of pH value in presence of *L*-malic acid
(c (substrate) = 1.56-500 mmol L^{-1},
c (NAD^+) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1}, pH 6.5)

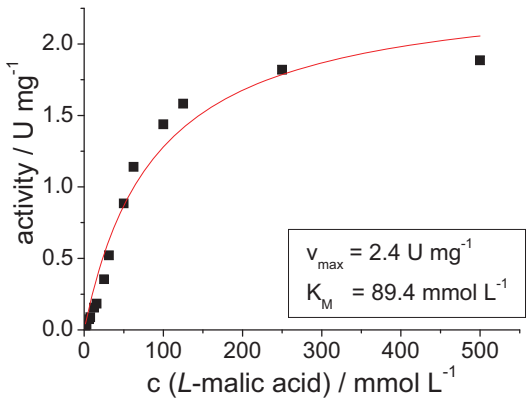


Figure 3-87 Effect of pH and $MnCl_2$ in presence of *L*-malic acid
(c (substrate) = 1.56-500 mmol L^{-1},
c (NAD^+) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1},
c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.5)

As well as the *L*-malic acid concentration the NAD^+ concentration has an effect on enzyme activity. At pH 6.0 at 20 mmol L^{-1} *L*-malic acid a v_{max} of 0.02 U mg^{-1} is observed (Figure 3-88). Maximum activity increases by a factor of 7.5 to 0.15 U mg^{-1} in the presence of 1.0 mmol L^{-1} $MnCl_2$ (Figure 3-89). An increase in *L*-malic acid concentration to 60 mmol L^{-1} has a positive effect on v_{max} as well (Figure 3-90). It increases by a factor of 2.5 to 0.05 U mg^{-1}. Addition of $MnCl_2$ affects v_{max} even more significantly than in presence of 20 mmol L^{-1} *L*-malic acid (Figure 3-91). v_{max} increases by a factor of 52 to 2.6 U mg^{-1}.

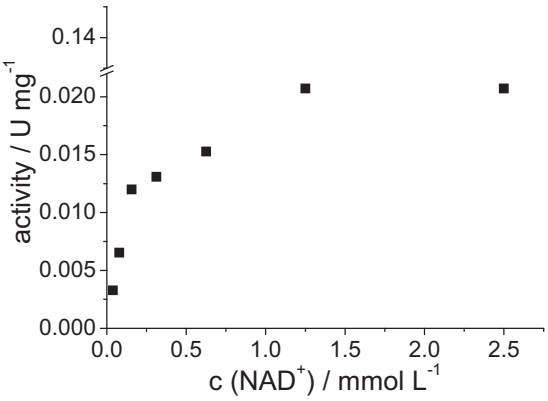


Figure 3-88 Variation of NAD^+ concentration
(c (NAD^+) = 0.04-2.5 mmol L^{-1},
c (*L*-malic acid) = 20 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

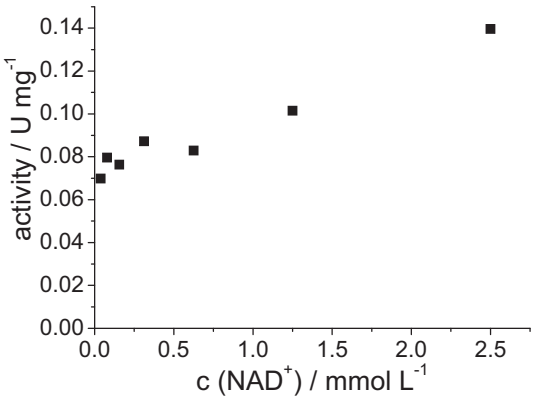


Figure 3-89 Effect of $MnCl_2$ in presence of NAD^+
(c (NAD^+) = 0.04-2.5 mmol L^{-1},
c (*L*-malic acid) = 20 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1},
c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.0)

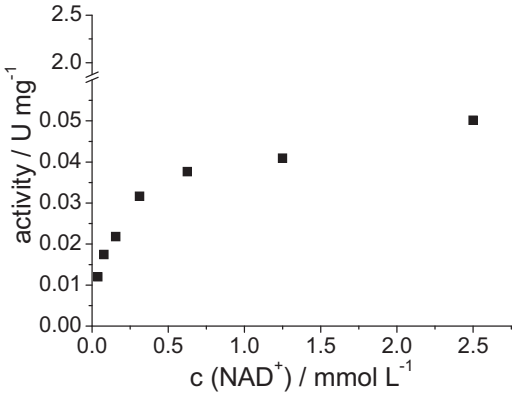


Figure 3-90 Effect of *L*-malic acid concentration in presence of NAD^+ (c (NAD^+) = 0.04-2.5 mmol L^{-1}, c (*L*-malic acid) = 60 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

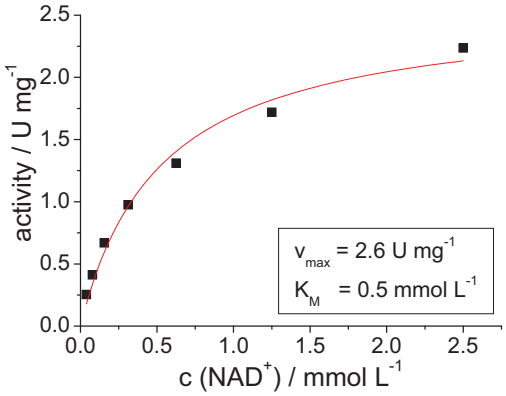


Figure 3-91 Effect of *L*-malic acid concentration and $MnCl_2$ in presence of NAD^+ (c (NAD^+) = 0.04-2.5 mmol L^{-1}, c (*L*-malic acid) = 60 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.0)

$MnCl_2$ has a positive effect on enzyme activity. Without the addition of Mn^{2+} ions at 20 mmol L^{-1} *L*-malic acid hardly any activity can be measured (Figure 3-92). The presence of $MnCl_2$ significantly increases MDH activity. Depending on the $MnCl_2$ concentration a limiting activity of about 1.2 U mg^{-1} is observed. An increase in *L*-malic acid concentration to 60 mmol L^{-1} changes the course of the activity-concentration plot when varying the $MnCl_2$ concentration (Figure 3-93). Activity increases towards its maximum of 1.75 U mg^{-1} at 1.6 mmol L^{-1}. v_{max} is reached at a higher $MnCl_2$ concentration than at 20 mmol L^{-1} *L*-malic acid. The results show that depending on the initial substrate concentration different $MnCl_2$ concentrations have to be applied to get optimum enzyme activity.

Since it is not possible to tell from the conducted measurements whether it is the MDH or the cofactor which is influenced by Mn^{2+} ions, either the MDH or the oxidized cofactor is incubated with 1.0 mmol L^{-1} $MnCl_2$ at 30 °C for a definite time before the reaction is started. At a *L*-malic acid concentration of 20 mmol L^{-1} the initial activity of the MDH decreases the longer the incubation time of the cofactor is (Figure 3-94). The highest activity of 0.056 U mg^{-1} is found after 0.5 min incubation and decreases to 0.045 U mg^{-1} after 26.5 min of incubation. In contrast, at 60 mmol L^{-1} *L*-malic acid concentration longer incubation times have a positive effect on enzyme activity (Figure 3-95). After 0.5 min activity is 1.0 U mg^{-1} and proceeds to 1.2 U mg^{-1} after 26.5 min. Obviously, at 20 mmol L^{-1} the *L*-malic acid concentration is the limiting value for the activity. Therefore, the effect of increasing $MnCl_2$ concentration cannot be monitored properly.

The incubation of the MDH with $MnCl_2$ at 60 mmol L^{-1} *L*-malic acid concentration also leads to an increase in activity with incubation time (Figure 3-96). After 0.5 min activity is 1.4 U mg^{-1} and increases to 1.51 U mg^{-1} after 21 min. Since the incubation of the MDH with $MnCl_2$ is more effective in terms of enzyme activity it may be beneficial for a reaction using enzyme dependent cofactor regeneration to incubate the MDH with Mn^{2+} ions before starting the reaction to allow for a fast supply with reduced cofactor. The reason for enhanced activity is believed to be the formation of a complex between the Mn^{2+} ion and the phosphorus atoms of the cofactor.[110-112]

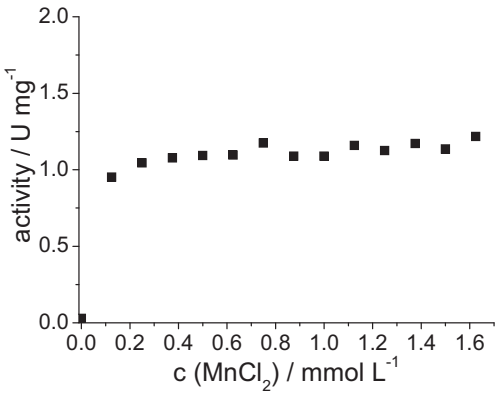


Figure 3-92 Variation of $MnCl_2$ concentration (c ($MnCl_2$) = 0-1.625 mmol L^{-1}, c (NAD^+) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 20 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

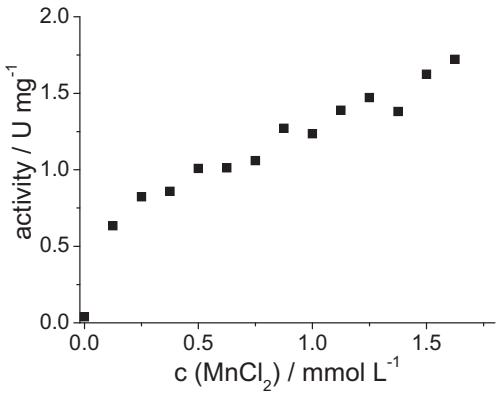


Figure 3-93 Effect of pH value in presence of $MnCl_2$ concentration (c ($MnCl_2$) = 0-1.625 mmol L^{-1}, c (NAD^+) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 20 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, pH 6.5)

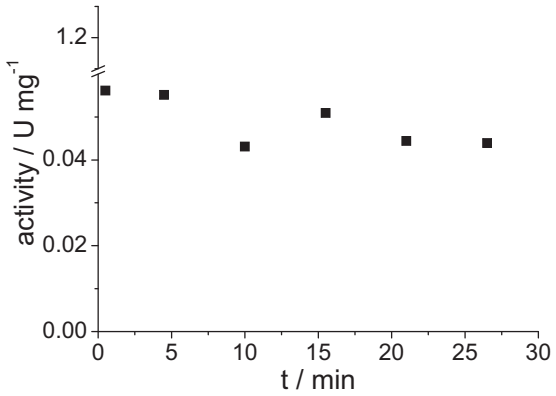


Figure 3-94 Incubation of NAD^+ with $MnCl_2$ at 20 mmol L^{-1} *L*-malic acid concentration (c (NAD^+) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 20 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.0)

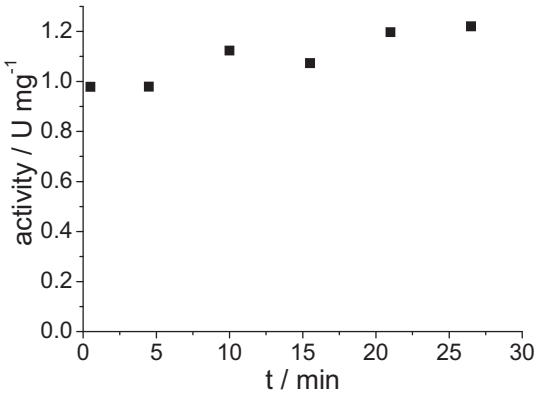


Figure 3-95 Incubation of NAD^+ with $MnCl_2$ at 60 mmol L^{-1} *L*-malic acid concentration (c (NAD^+) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 60 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.0)

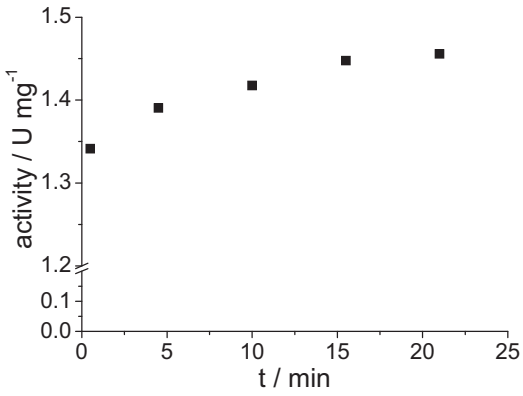


Figure 3-96 Incubation of MDH with $MnCl_2$ at 60 mmol L^{-1} *L*-malic acid concentration at pH 6.5 (c (NAD^+) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 60 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, c ($MnCl_2$) = 1.0 mmol L^{-1}, pH 6.0)

3.6 Kinetic Characterization of the NADP-dependent MDH

The NADP-dependent MDH is kinetically characterized by investigating the dependence of enzyme activity on:

- concentration of *L*-malic acid
- concentration of $NADP^+$
- concentration of $MgCl_2$

For the activity depending on the *L*-malic acid concentration substrate inhibition is found (Figure 3-97). Activity increases to v_{max} of 0.3 U mg^{-1} and decreases exponentially afterwards. K_M of 9.7 mmol L^{-1} shows a high affinity of the MDH towards *L*-malic acid. The constant for substrate inhibition K_S is 38.6 mmol L^{-1}. The dependence of the MDH activity on the $NADP^+$ concentration shows a maximum activity of 0.24 U mg^{-1} reached at 0.63 mmol L^{-1} $NADP^+$ (Figure 3-98). This allows for fast cofactor regeneration at low $NADP^+$ concentrations. Since for the NAD-dependent MDH it was observed that the presence of 1.0 mmol L^{-1} $MnCl_2$ increases enzyme activity significantly, the dependence of the activity of NADP-dependent MDH on Mn^{2+} ions is investigated as well. $MnCl_2$ has a positive effect on MDH activity and increases it significantly (Figure 3-99). Already low Mn^{2+} concentrations lead to a 28-fold increase in activity.

As it was found for the NAD-dependent MDH that the cofactor forms a complex with Mn^{2+} ions the same question has to be answered for the NADP-dependent MDH. Incubation of the MDH with 1.0 mmol L^{-1} $MnCl_2$ causes an increase in enzyme activity the longer the incubation time is (Figure 3-100). Compared to 0.007 U mg^{-1} without $MnCl_2$ (Figure 3-97) activity increases to 0.17 U mg^{-1} after 0.5 min incubation and reaches 0.2 U mg^{-1} after 21 min. When incubating $NADP^+$ with Mn^{2+} ions the increase is more significant starting at 0.17 U mg^{-1} after 0.5 min and reaching 0.3 U mg^{-1} after 26 min (Figure 3-101). It is believed that the cofactor forms a complex with Mn^{2+} ions which leads to a significant increase in enzyme activity.[110-112]

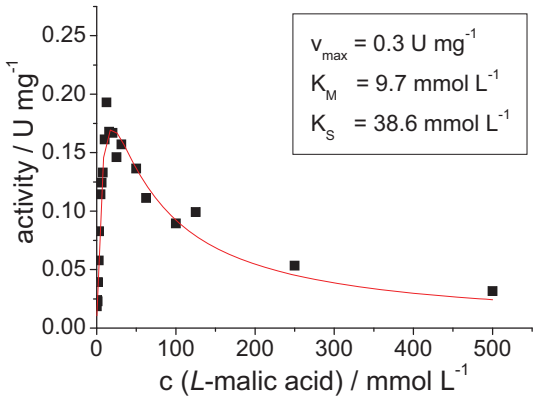


Figure 3-97 Variation of *L*-malic acid concentration
(c (substrate) = 0.31-500 mmol L^{-1},
c ($NADP^+$) = 0.5 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

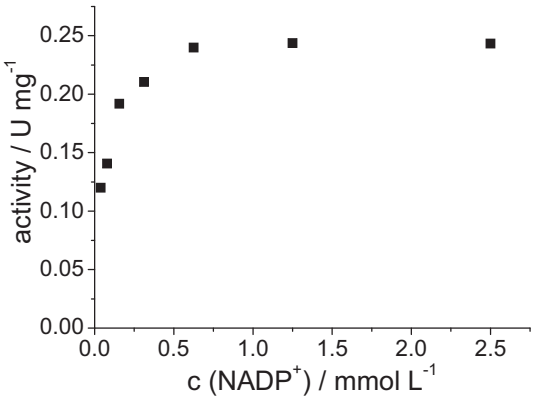


Figure 3-98 Variation of $NADP^+$ concentration
(c ($NADP^+$) = 0.04-2.5 mmol L^{-1},
c (*L*-malic acid) = 20 mmol L^{-1},
c (buffer) = 50 mmol L^{-1},
c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

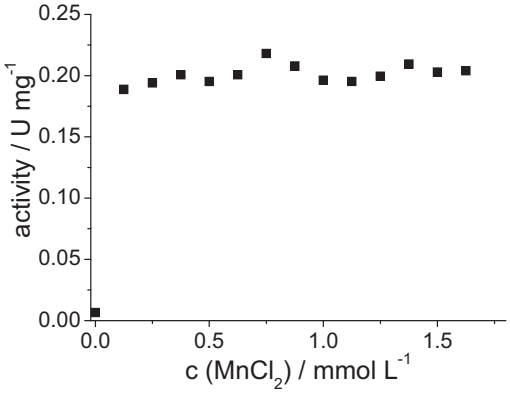


Figure 3-99 Variation of $MnCl_2$ concentration (c ($MnCl_2$) = 0-1.63 mmol L^{-1}, c ($NADP^+$) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 20 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

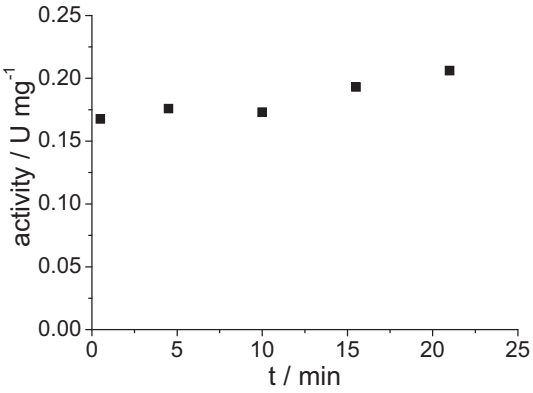


Figure 3-100 Incubation of MDH with $MnCl_2$ (c ($NADP^+$) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 20 mmol L^{-1}, c ($MnCl_2$) = 1.0 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

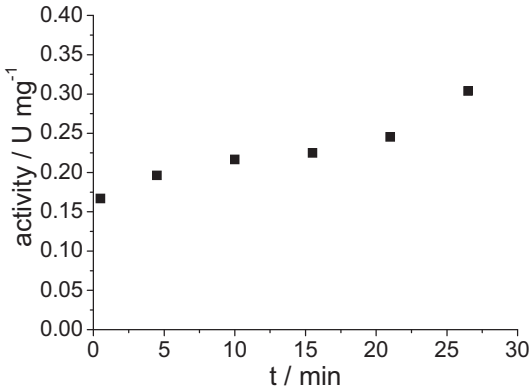


Figure 3-101 Incubation of $NADP^+$ with $MnCl_2$ (c ($NADP^+$) = 0.5 mmol L^{-1}, c (*L*-malic acid) = 20 mmol L^{-1}, c ($MnCl_2$) = 1.0 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, c (MDH) = 0.1 mg mL^{-1}, pH 6.0)

3.7 Summary of Kinetic Characterization

The SADH-X2 and the two preparations of the LB-ADH both show activity towards the chosen aliphatic and aromatic ketones. The SADH-X2 provides highest activity towards all ketones except towards 2-butanone. Here, the solid LB-ADH preparation is more active. For both the SADH-X2 and the solid LB-ADH activity is dependent on the chain length and on the size of the R-groups. The liquid LB-ADH preparation shows high activity towards all ketones, but no strong dependence on the molecule structure is observed. For the SADH-X2 highest

activity of 13.7 U mg^{-1} (Figure 3-4) is found towards 2-heptanone while the solid and the liquid LB-ADH both show highest activity of 8.2 U mg^{-1} and 620.5 U mL^{-1}, respectively, in presence of *p*-chloro acetophenone (Figure 3-7 and Figure 3-9).
The characterization of the three enzymes shows activity for 2-butanone as well as for all tested substrates depending on reaction conditions. The activity measurements resulted in the following overall highest activities for each enzyme:

- SADH-X2: 10.0 U mg^{-1} (2-butanone, 50 mmol L^{-1} buffer, 1.0 mmol L^{-1} $MgCl_2$)
- solid LB-ADH: 9.7 U mg^{-1} (acetophenone, 50 mmol L^{-1} buffer, 1.0 mmol L^{-1} $MgCl_2$)
- liquid LB-ADH: 890.6 U mL^{-1} (acetone, 50 mmol L^{-1} buffer)

The activity measurements allowed for the identification of the main influence factors on ADH activity. These are:

- preparation and purification of the ADH
- substrate
- saturation with MTBE
- presence of $MgCl_2$
- buffer concentration

For each ADH activity depends on the substrate, and depending on the substrate activity depends on the reaction conditions. No general conclusions can be drawn. The cross dependence of the influencing factors is complex, and the contribution of individual factors cannot be easily separated. A general explanation is not possible. Therefore, the actual kinetic behavior of a reaction system is not predictable. Concentrations of substrate, product, co-substrate, co-product, and oxidized and reduced cofactor change continuously.

The two different types of MDH, the NAD-dependent and the NADP-dependent, both show good activity to the oxidized and reduced cofactors. Activity is strongly dependent on the concentrations of the cofactors, the different substrates like 2-butanone, *rac*-2-butanol, acetone, and 2-propanol, and on the concentration of *L*-malic acid. Especially the presence of $MnCl_2$ highly improves enzyme activity. Activities are measured in the range of 0.005 U mg^{-1} to 2.2 U mg^{-1} for the NAD-dependent MDH, and between 0.02 U mg^{-1} and 0.3 U mg^{-1} for the NADP-dependent MDH. By incubation experiments it was proven that the cofactor forms a complex with Mn^{2+} ions which improves enzyme activity significantly.

The results of the kinetic characterization show that it is not possible to conclude from one enzyme to another or from one preparation of the same enzyme to another preparation. Therefore, for every specific reaction system the kinetic background has to be investigated to find reaction conditions which can be used for batch synthesis. The search strategy for the screening should include an array of actual reaction conditions. Furthermore, selectivity is not taken into account for the kinetic characterization. It will be specially dealt with during the batch and continuous experiments.

4 Synthesis of Chiral Alcohols using One-Phase Systems

4.1 Batch Reactions

4.1.1 Introduction

Based on the results obtained from the kinetic characterization reaction conditions for batch experiments in one-phase systems are chosen. In certain time intervals samples are taken and analyzed using chiral GC. From the chromatograms conversion and *ee* are calculated and plotted against time. The experiments will display the dependence of conversion and *ee* on reaction conditions. From the batch experiments the conditions for two-phase reaction systems will be chosen and optimized to enable experiments on preparative scale.
The batches are kinetically characterized by calculating the initial reaction rates from the conversion against time plots. Due to the different composition of the reaction mixture and its complexity compared to the kinetic assays it is not possible to directly compare the kinetic behavior of the assays to that of the batch experiments.

4.1.2 Batch Reactions using the SADH-X2

The SADH-X2 is employed for the batch synthesis of (*S*)-2-butanol in a one-phase system using substrate and enzyme dependent cofactor regeneration. At a 2-butanone concentration of 20 mmol L^{-1} and a 2-propanol concentration of 0.2 mol L^{-1} maximum conversion of 11% is observed after 1 h reaction time and does not change within the following 14 h (Figure 4-1). The enantiomeric excess decreases from 80% to 3%. The reaction is again carried out at a co-substrate concentration of 2.0 mol L^{-1} resulting in a different behaviour of conversion and *ee* (Figure 4-2). Conversion reaches its equilibrium value of 1.2% after 1.5 h and is constant over 15 h. Initial *ee* is 83%. Within the given reaction time it decreases to 75% which is 69% less decrease than at the lower co-substrate concentration. Obviously, at a 200-fold excess of 2-propanol the ADH does not convert the product alcohol as fast as at a 20-fold excess, but the high solvent concentration seems to inhibit the ADH which leads to a lower conversion.
Since *ee* and conversion are not satisfying when using the SADH-X2 with substrate dependent cofactor regeneration, similar reactions are carried out using enzyme dependent cofactor regeneration. The NAD-dependent MDH is employed with 30 mmol L^{-1} *L*-malic acid using different MDH:ADH ratios. At a ratio of 2.5:1 maximum conversion of 41% is reached after 3.2 h reaction time and decreases to 22% within the following 4 h (Figure 4-3) Initial *ee*

is 91% (*S*) and decreases linearly to 11% (*R*) at a rate of 14% h^{-1}. The concentrations of MDH and *L*-malic acid are too low to ensure a sufficient regeneration of the cofactor. Therefore, the ADH oxidizes the product alcohol instead of reducing the ketone. Another reaction is done using 5 mg mL^{-1} of each enzyme (MDH:ADH = 1:1) and 60 mmol L^{-1} *L*-malic acid (Figure 4-4). Maximum conversion of 73% is reached after 1.4 h reaction time and decreases linearly to 32% within the following 4 h at a rate of 10.3% h^{-1}. Initial *ee* is 83% (*S*) and decreases exponentially to 13% (*R*). Maximum conversion is reached after a shorter reaction time and is 32% higher than for the reaction using a MDH:ADH ratio of 2.5:1, but the decrease in conversion is higher and initial *ee* is 8% lower. A further increase in ADH and MDH concentration to 8 mg mL^{-1} leads to a maximum conversion of 78% after 0.7 h. It decreases exponentially to 31% after 6 h (Figure 4-5). Initial *ee* is 50% and decreases exponentially to a racemic mixture which is reached after about 2 h. The obtained results show that with enzyme dependent cofactor regeneration it is possible to achieve higher conversion and *ee* compared to substrate dependent cofactor regeneration, but it is challenging to find the optimum reaction conditions with two enzymes and two different substrates in one reaction system. The number of parameters which can and need to be adjusted and which influence enzyme activity and selectivity is >10.

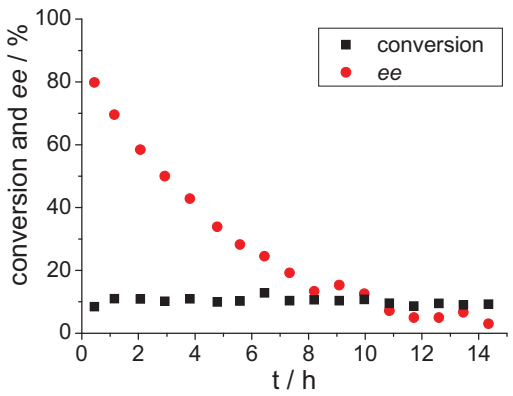


Figure 4-1 Batch synthesis of (*S*)-2-butanol
(c (2-butanone) = 20 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (buffer) = 100 mmol L^{-1},
V (reaction) = 1.0 mL, experiment: PM 154)

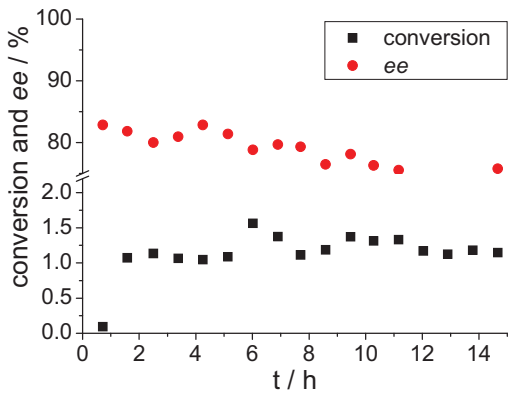


Figure 4-2 Batch synthesis of (*S*)-2-butanol
(c (2-butanone) = 20 mmol L^{-1},
c (2-propanol) = 2.0 mol L^{-1},
c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (buffer) = 100 mmol L^{-1},
V (reaction) = 1.0 mL, experiment: PM 155)

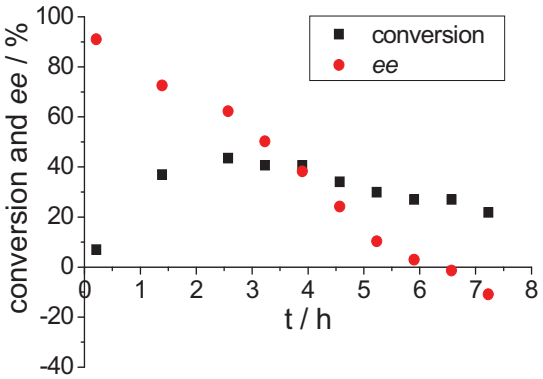


Figure 4-3 Batch synthesis of (*S*)-2-butanol
(c (2-butanone) = 25 mmol L^{-1},
c (*L*-malic acid) = 30.0 mmol L^{-1},
c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (MDH) = 2.5 mg mL^{-1},
c (buffer) = 100 mmol L^{-1},
V (reaction) = 1.0 mL, experiment: TH 01)

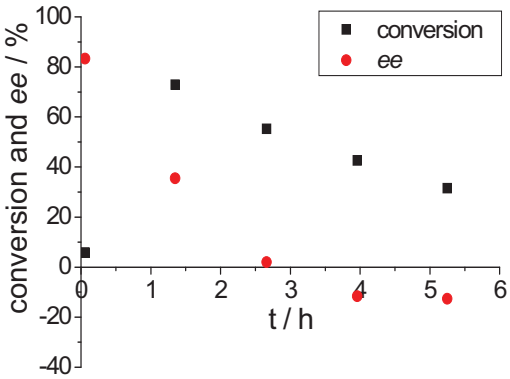


Figure 4-4 Batch synthesis of (*S*)-2-butanol
(c (2-butanone) = 25 mmol L^{-1},
c (*L*-malic acid) = 60.0 mmol L^{-1},
c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 5.0 mg mL^{-1},
c (MDH) = 5.0 mg mL^{-1},
c (buffer) = 100 mmol L^{-1},
V (reaction) = 1.0 mL, experiment: TH 02)

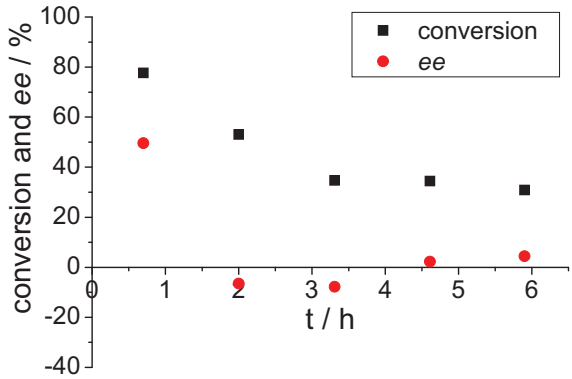


Figure 4-5 Batch synthesis of (*S*)-2-butanol
(c (2-butanone) = 25 mmol L^{-1}, c (*L*-malic acid) = 60.0 mmol L^{-1}, c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 8.0 mg mL^{-1}, c (MDH) = 8.0 mg mL^{-1}, c (buffer) = 100 mmol L^{-1}, V (reaction) = 1.0 mL,
experiment: TH 03)

4.1.3 Batch Reactions using the LB-ADH

The LB-ADH is utilized to synthesize (*R*)-2-butanol in a one-phase system (Table 4-1). Conversion and selectivity are investigated depending on the 2-butanone and the 2-propanol concentration using substrate dependent cofactor regeneration. Generally, the initial reaction rate shows an increase at higher substrate concentration and also higher values with a

10-fold increase of the 2-propanol concentration (Figure 4-6). Obviously, the increase of the co-substrate concentration leads to better supply of NADPH, and, therefore, has a positive effect on the initial reaction rate. In contrast to the SADH-X2 equilibrium conversion is hardly affected.

At a 2-butanone concentration of 20 mmol L^{-1} conversion does not reach equilibrium within the investigated time range at a 2-propanol concentration of 0.2 mol L^{-1} (Figure 4-8). Comparing the conversion-time plot with that of the same reaction carried out at 2.0 mol L^{-1} co-substrate concentration (Figure 4-9) shows that equilibrium conversion of the reaction with less 2-propanol may reach a higher conversion. Since for both reactions conversion is below 10% GC results regarding the detection of the (*S*)-enantiomer are not reliable. It is expected that racemisation is faster at lower co-substrate concentrations.

At a 2-butanone concentration of 50 mmol L^{-1} conversion is higher at 2.0 mol L^{-1} 2-propanol (Figure 4-10) than at 0.2 mol L^{-1} (Figure 4-9, Table 4-2) after 2 h reaction time. At the lower co-substrate concentration conversion reaches its maximum value of 0.4%. At 2.0 mol L^{-1} 2-propanol equilibrium conversion is 2.9% (Table 4-2). The initial reaction rates are the same with 0.004 mmol L^{-1} (Table 4-2).

At 100 mmol L^{-1} 2-butanone concentration the initial reaction rate is about four times higher at the higher co-substrate concentration than at the lower concentration (Figure 4-11 and Figure 4-12, Table 4-2). The same applies for the maximum conversion. For both 2-propanol concentrations experimental equilibrium conversion is reached. It is 1.3% at 0.2 mol L^{-1} and 5.5% at 2.0 mol L^{-1} co-substrate concentration (Table 4-2).

Regarding the racemisation rate there is an apparent dependence on the 2-butanone concentration as well as on the conversion (Figure 4-7). With increasing substrate concentration racemisation rate decreases significantly since the enzyme is better saturated with ketone and, therefore, does not re-oxidize and racemize the product alcohol as fast as at lower concentrations. This leads to lower initial *ee* with increasing 2-butanone concentration (Table 4-3). Due to the similarity of the product alcohol and the co-substrate, in presence of low 2-propanol concentrations the favored (*R*)-alcohol will be used for the cofactor regeneration (Table 4-3).

With increasing substrate concentration maximum conversion decreases, but the absolute product concentration increases. With increasing co-substrate concentration conversion increases at constant substrate concentration. For the *ee* the values principally are positively influenced by increasing substrate concentration, but with increasing 2-propanol concentration maximum conversion increases as well.

Table 4-1 Overview of experiments for the synthesis of (*R*)-2-butanol in a one-phase system using the solid LB-ADH

c (2-butanone) / mmol L^{-1}	experiment c (2-propanol) / mol L^{-1}	
	0.2	2.0
20	PM 174	PM 178
50	AL 8	AL 9
100	PM 179	PM 180

Table 4-2 Conversion and initial reaction rates of batchwise synthesis of (*R*)-2-butanol in a one-phase system using the solid LB-ADH

c (2-butanone) / mmol L^{-1}	max conversion / % c (2-propanol) / mol L^{-1}		initial reaction rate / mmol L^{-1} min^{-1} c (2-propanol) / mol L^{-1}	
	0.2	2.0	0.2	2.0
20	8.8	7.5	0.012	0.110
50	0.4	2.9	0.004	0.004
100	1.3	5.5	0.100	0.440

Table 4-3 Initial *ee* and racemisation rates of batchwise synthesis of (*R*)-2-butanol in a one-phase system using the solid LB-ADH

c (2-butanone) / mmol L^{-1}	max *ee* / % c (2-propanol) / mol L^{-1}		racemisation rate / % min^{-1} c (2-propanol) / mol L^{-1}	
	0.2	2.0	0.2	2.0
20	>99.0	33.0 15.0 (*S*)	-	3.0
50	19.5	65.3	0.3	0.6
100	14.3 (*R*) 45.5 (*S*)	42.9	0.3	0.4

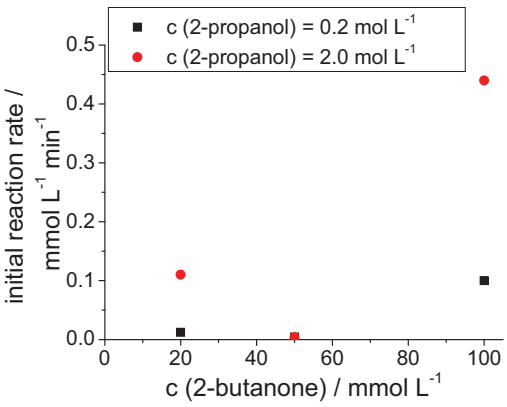


Figure 4-6 Initial reaction rates of batchwise synthesis of (*R*)-2-butanol in a one-phase system using the solid LB-ADH

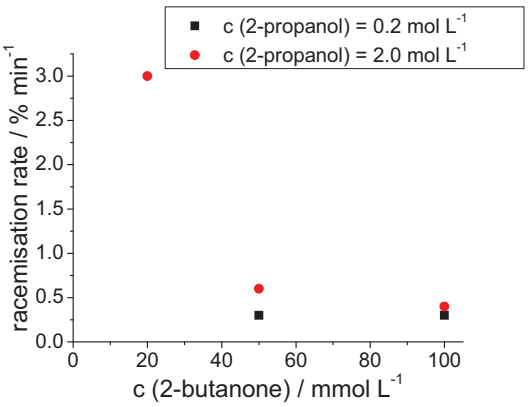


Figure 4-7 Racemisation rates of batchwise synthesis of (*R*)-2-butanol in a one-phase system using the solid LB-ADH

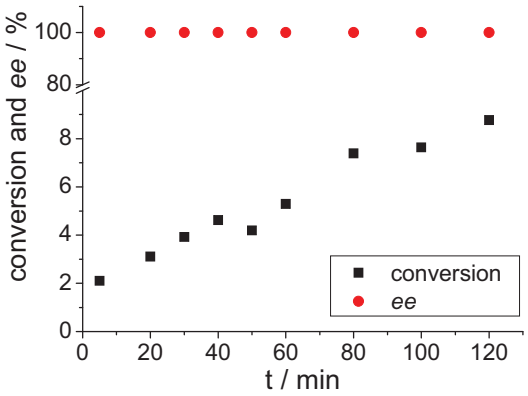


Figure 4-8 Batch synthesis of (*R*)-2-butanol
(c (2-butanone) = 20 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 174)

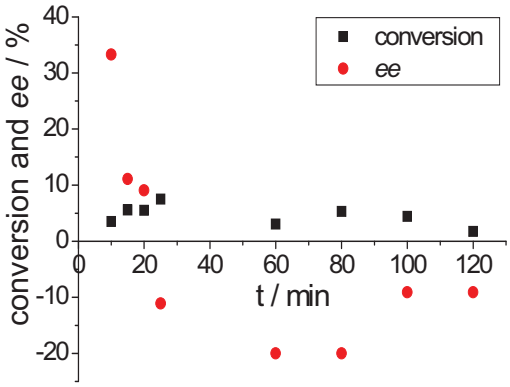


Figure 4-9 Batch synthesis of (*R*)-2-butanol
(c (2-butanone) = 20 mmol L^{-1},
c (2-propanol) = 2.0 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 178)

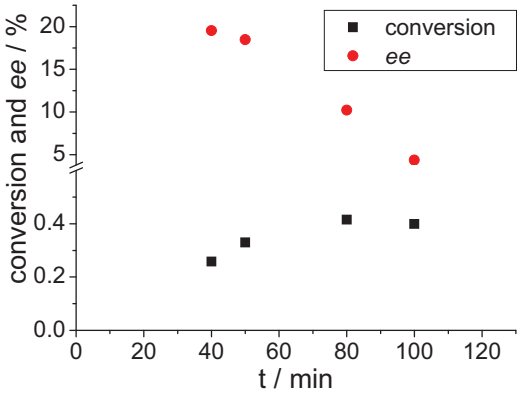


Figure 4-10 Batch synthesis of (*R*)-2-butanol
(c (2-butanone) = 50 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: AL 8)

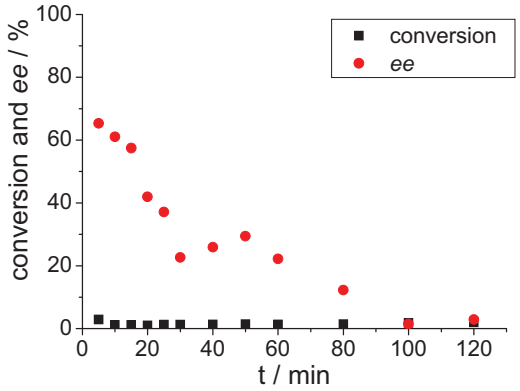


Figure 4-11 Batch synthesis of (*R*)-2-butanol
(c (2-butanone) = 50 mmol L^{-1},
c (2-propanol) = 2.0 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: AL 9)

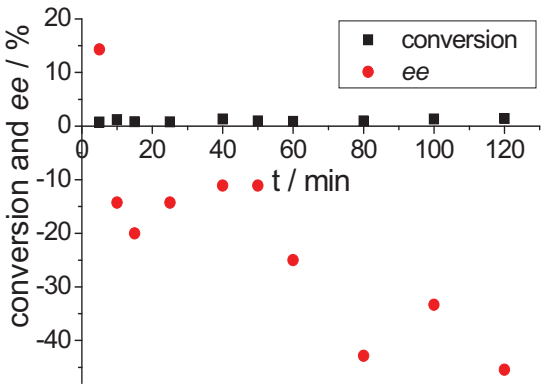


Figure 4-12 Batch synthesis of (*R*)-2-butanol
(c (2-butanone) = 100 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 179)

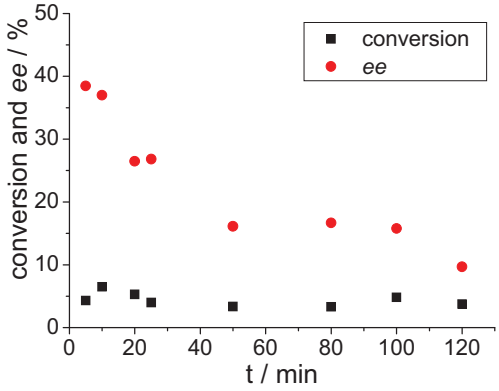
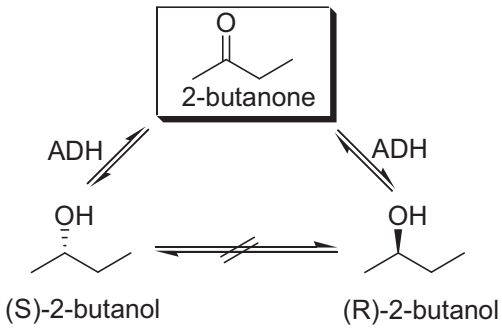


Figure 4-13 Batch synthesis of (*R*)-2-butanol
(c (2-butanone) = 100 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 180)

4.1.4 Racemisation Mechanism of the LB-ADH and the SADH-X2

When synthesizing enantiopure 2-butanol by reducing the prochiral ketone 2-butanone using the SADH-X2 or the LB-ADH the problem of product racemisation occurs (Chapters 4.1.2 and 4.1.3). Therefore, the mechanism of racemisation has to be investigated. Different pathways are possible. One is the direct racemisation between (*R*)- and (*S*)-2-butanol, the other one is racemisation via re-oxidation of the product alcohol or oxidation by exposure to air (Scheme 4-1).



Scheme 4-1 Racemisation mechanism of 2-butanol enantiomers

To investigate the possibility of racemisation via oxidation by air enantiopure (*R*)-2-butanol is incubated for 2 h in a 50 mmol L^{-1} buffer at pH 6.5 at room temperature. Within 2 h incubation

time *ee* does not change. Therefore, racemisation of the product alcohol must occur via 2-butanone as intermediate (Scheme 4-1). The next step is the investigation of the oxidation of (*R*)-2-butanol utilizing the solid LB-ADH under reductive conditions, i.e. to apply the reaction conditions used for the synthesis of the (*R*)-alcohol in a one-phase system. But instead of 2-butanone 10 mmol L^{-1} (*R*)-2-butanol are added. The plot of conversion and *ee* against time shows the conversion of the (*R*)-alcohol to 2-butanone and the *ee* pro-(*R*), whereas a negative *ee* stands for an enantiomeric excess pro-(*S*) (Figure 4-14). Conversion of the enantiopure alcohol is very fast and is >99% 5 min after the reaction is started. The initial *ee* is 40% pro-(*S*) and increases to 50%. The results show that the conversion of (*R*)-2-butanol is nearly quantitative and that the decrease in *ee* does not stop at the racemic mixture but heads towards an *ee* pro-(*S*). Therefore, another experiment is conducted to investigate the behavior of the LB-ADH in presence of *rac*-2-butanol (Figure 4-15). After 5 min reaction time conversion is 70% and increases to >99% within 20 min. The *ee* increases from racemic to 70% pro-(*S*) within the first 5 min reaction time and increases even further to 95% pro-(*S*). The reaction proceeds slower than in the presence of (*R*)-2-butanol, but the (*S*)-enantiomer is even more favored since already 70% (*S*)-enantiomer are present at the beginning of the reaction. The question occurring is what the redox equivalent assimilating the hydride generated in the oxidation reaction is and where it has its origin. Taking the initial $NADP^+$ concentration of 0.1 mmol L^{-1} into account would only allow for a conversion of 1%. To answer the question the oxidation of (*R*)-2-butanol is conducted without addition of $NADP^+$ (Figure 4-16). The (*R*)-alcohol is quantitatively converted to 2-butanone. Initial enantiomeric excess is 45% after 5 min reaction time and increases slightly to 65% after 120 min. Compared to the same reaction in presence of $NADP^+$ (Figure 4-14) the absence of the cofactor has a positive effect on the *ee*, but conversion is still quantitative. Due to the missing cofactor racemisation is less intensive, but the enzyme preparation must contain a redox equivalent which allows for the high conversion.

It may be possible that a second enzyme, e.g. an oxygenase is present in the solid enzyme preparation. To deactivate this undesired enzyme, the enzyme solution is incubated at 45 °C for 5 min before the reaction is started. This way, also less robust redox equivalents contained in the enzyme preparation should be deactivated. The LB-ADH should not be affected by the temperature treatment because it is tolerant towards moderate temperatures. The oxidation of (*R*)-2-butanol without $NADP^+$ using the heated enzyme solution is still quantitative (Figure 4-17), but the initial *ee* is 86% and does not change over 120 min reaction time. The initial *ee* is 41% higher than for the same experiment conducted with the non-heated enzyme solution. Obviously, the enzyme preparation contains some redox equivalent or a racemase which can partly be deactivated by treating the dissolved lyophilisate at moderate temperatures.

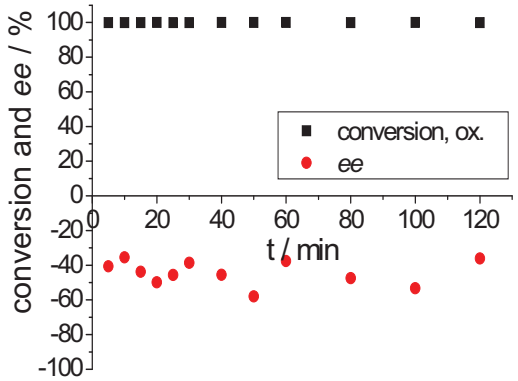


Figure 4-14 Oxidation of (*R*)-2-butanol
(c ((*R*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 0.25 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: TH 04)

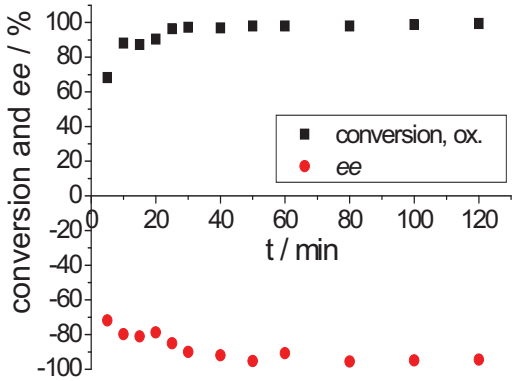


Figure 4-15 Oxidation of *rac*-2-butanol
(c (*rac*-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 0.25 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: TH 05)

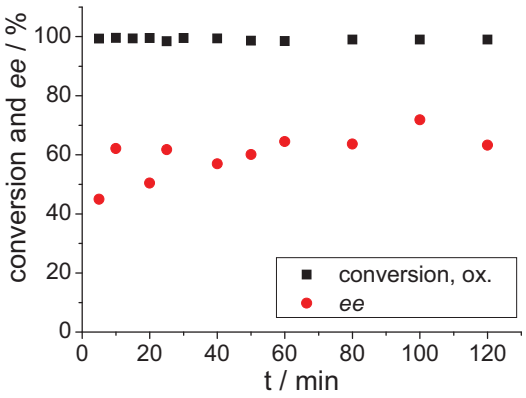


Figure 4-16 Oxidation of (*R*)-2-butanol
without $NADP^+$
(c ((*R*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (LB-ADH) = 0.25 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: TH 06)

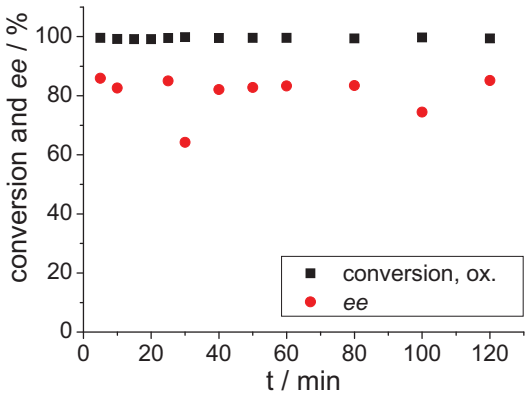


Figure 4-17 Oxidation of (*R*)-2-butanol
without $NADP^+$
(c ((*R*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (LB-ADH) = 0.25 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
enzyme heated to 45 °C for 5 min,
experiment: TH 07)

The described experiments are repeated with the liquid LB-ADH preparation which was purified by column chromatography. The oxidation of (*R*)-2-butanol in presence of $NADP^+$ has an initial conversion of 18% after 15 min reaction time (Figure 4-18), and it increases to 80% within 120 min. Initial *ee* is 85% pro-(*R*). Racemisation still occurs, but *ee* is still 28%

pro-(*R*) after 120 min reaction time. The same positive effect of the enzyme purification is revealed by the oxidation of *rac*-2-butanol (Figure 4-19). After 5 min reaction time conversion is 37% and increases slightly to 48% after 120 min. The *ee* is about 93% and is in the same range as for the same experiment conducted with the non-purified LB-ADH (Figure 4-15), but conversion is still higher than the $NADP^+$ concentration does allow for.

When no $NADP^+$ is added to the reaction mixture the purified LB-ADH does not convert the (*R*)-2-butanol to 2-butanone (Figure 4-20), and enantiomeric excess is 90% and does not change over reaction time. The enantiomeric excess of racemic 2-butanol does not change when using the purified LB-ADH without addition of $NADP^+$ (Figure 4-21), and no conversion can be observed.

The conducted experiments show that, obviously, the non-purified LB-ADH preparation contains an oxidative equivalent which can be removed by column chromatography, and which is responsible for the heavy decrease in *ee*. Furthermore, the results of the experiments proof that the racemisation of the (*R*)-alcohol is not a direct racemisation but that 2-butanone is the intermediate (Scheme 4-1).

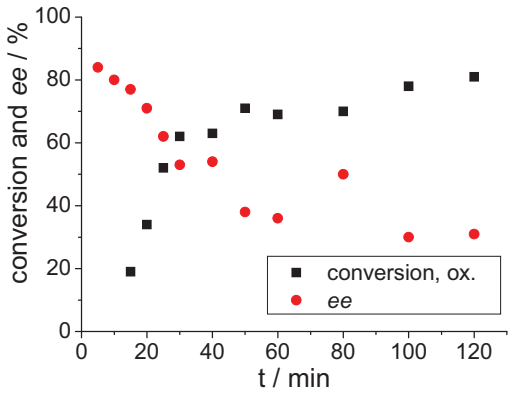


Figure 4-18 Oxidation of (*R*)-2-butanol
(c ((*R*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 2.5 µL mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 197)

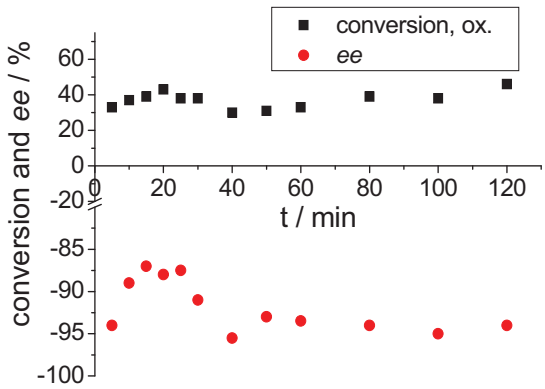


Figure 4-19 Oxidation of *rac*-2-butanol
(c (*rac*-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c ($NADP^+$) = 0.1 mmol L^{-1},
c (LB-ADH) = 2.5 µL mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 198)

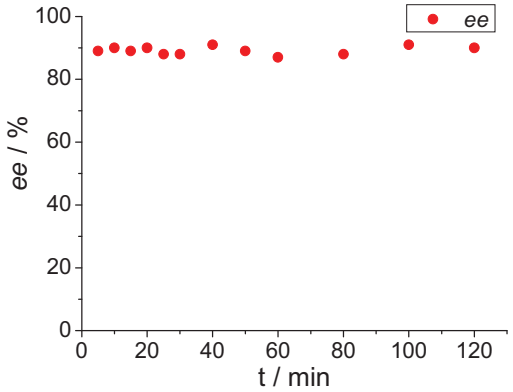


Figure 4-20 Oxidation of (*R*)-2-butanol without $NADP^+$
(c ((*R*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (LB-ADH) = 2.5 µL mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 199)

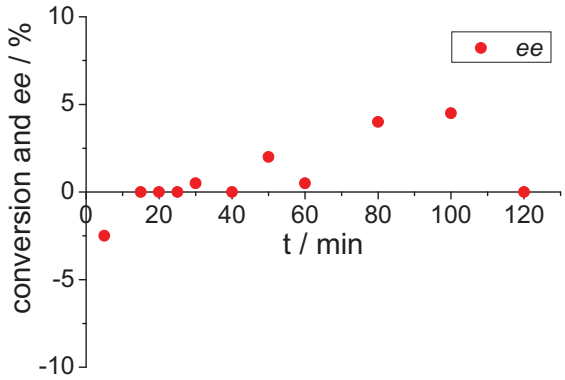


Figure 4-21 Oxidation of *rac*-2-butanol without $NADP^+$
(c (*rac*-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (LB-ADH) = 2.5 µL mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: PM 200)

Since the results obtained for the LB-ADH show that a special purification step is necessary to suppress decrease in enantiomeric excess, similar experiments are conducted to investigate the racemisation behavior of the SADH-X2. (*S*)- and *rac*-2-butanol are used as substrates at reductive reaction conditions in presence and in absence of 0.1 mmol L^{-1} NAD^+. The oxidation of the (*S*)-alcohol starts at 6% conversion after 25 min reaction time and reaches its equilibrium conversion of 4% after 120 min (Figure 4-22). Obviously, no racemisation occurs. The *ee* of the pure (*S*)-alcohol is 80%. This value is also observed for the oxidation reaction and does not change over reaction time. In contrast, the racemic alcohol is converted to 2-butanone up to 20%, whereas the *ee* does not change (Figure 4-23). In absence of NAD^+ the conversion of (*S*)-2-butanol to 2-butanone reaches 20% while the *ee* is slightly higher than 80% and does not change (Figure 4-24). In contrast, the conversion of the racemic alcohol in absence of the cofactor reaches 30% without any change in enantiomeric excess (Figure 4-25). The SADH-X2 surprisingly achieves a higher conversion of (*S*)- and *rac*-2-butanol without NAD^+. This hints towards the presence of a redox equivalent contained in the enzyme preparation, but the effect is less significant than for the LB-ADH. The SADH preparation does not affect the enantiomeric excess of the alcohol and the re-oxidation is less distinct.

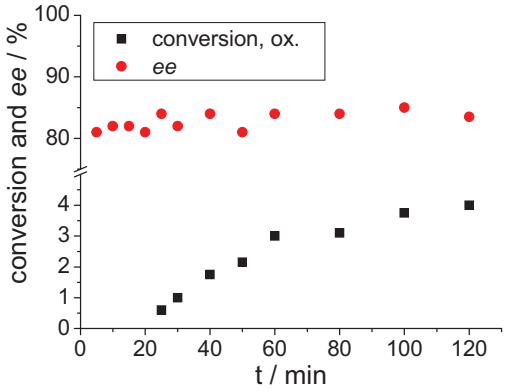


Figure 4-22 Oxidation of (*S*)-2-butanol
(c ((*S*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: SA 01)

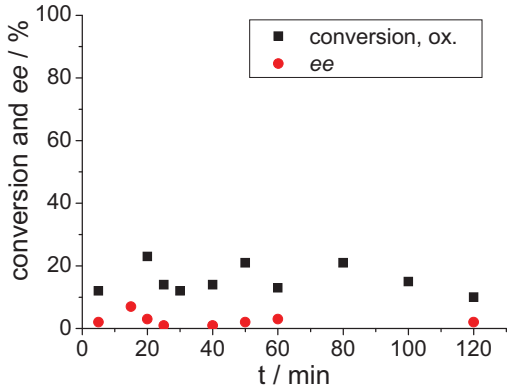


Figure 4-23 Oxidation of *rac*-2-butanol
(c (*rac*-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (NAD^+) = 0.1 mmol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: SA 02)

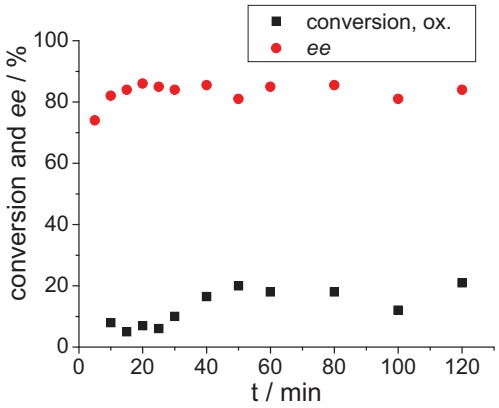


Figure 4-24 Oxidation of (*S*)-2-butanol without NAD^+
(c ((*S*)-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: SA 03)

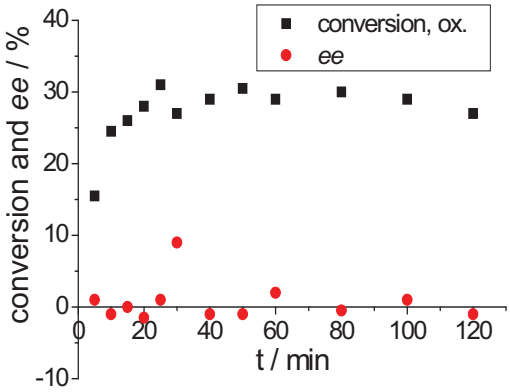


Figure 4-25 Oxidation of *rac*-2-butanol without NAD^+
(c (*rac*-2-butanol) = 10 mmol L^{-1},
c (2-propanol) = 0.2 mol L^{-1},
c (SADH-X2) = 1.0 mg mL^{-1},
c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL,
experiment: SA 04)

4.2 Continuous Reactions using the Enzyme Membrane Reactor

The continuous synthesis of (*S*)-2-butanol with the SADH-X2 in a one-phase system is performed using an enzyme membrane reactor with a total volume of 10 mL. The reactor

contains a membrane which holds back the enzyme. Cofactor regeneration is performed enzyme dependent using the NAD-dependent MDH and *L*-malic acid. An excess in MDH concentration is chosen (MDH:SADH-X2 2.5:2) since the batch experiments showed that it is necessary to ensure a sufficient supply with NADH to avoid re-oxidation and racemisation of the product alcohol. The reaction is started by addition of the ADH solution. At a flow rate of 0.45 mL min^{-1} (τ = 22.2 min) the reaction is run for 19 h (51.4 τ) (Figure 4-26). A steady state in conversion is reached after 5.4 h (16 τ). Initial conversion is 8% and is reduced to 1% at a rate of 1.3% min^{-1}. Initial enantiomeric excess is 72%. It decreases to 47% within 8 h and afterwards increases to 80% within the following 10 h. The *ee* does not show the fast racemisation observed for the corresponding batch experiments, but conversion is much lower. Therefore, a second continuous experiment is started at a lower flow rate of 0.36 mL min^{-1} (τ = 27.8 min) (Figure 4-27). The higher residence time is supposed to enable higher conversion. Initial conversion is 10% and decreases to 1.2% in the steady state which is reached after 3 h (6.5 τ) and maintained for 65 h (140.3 τ). With 97% the *ee* is high at the beginning of the reaction and is reduced to about 60% within 3 h (6.5 τ). After attaching a new substrate solution after 20 h (42.3 τ) *ee* increases slightly to 68% within 10 h (21.6 τ) and is constant at this value until the reaction is stopped after 68 h (146.8 τ) overall reaction time. The change in residence time influences the initial values of conversion and *ee* positively, but a change in equilibrium values is not observed. A reason for the decrease in conversion and the low equilibrium conversion may be an insufficient cofactor regeneration which leads to re-oxidation of the product because the ADH consumes the product alcohol for cofactor regeneration. Another reason for the drop in conversion may be a loss of enzyme. Since the SADH-X2 has a very small diameter[105] it may pass through the membrane. A leakage at the membrane may be responsible for loss of both enzymes as well. The conducted continuous experiments have a productivity for the ADH of 0.33 mmol mg^{-1} and 1.17 mmol mg^{-1}, respectively. They show that it is generally possible to perform continuous reactions in a one-phase system to synthesize small chiral alcohols. But the results also show that it is essential to optimize the reaction setup to obtain better results. Compared to batch reactions, selectivity of the reaction can be improved by the continuous process, but conversion reveals that a two-phase reaction system may be easier to handle due to integrated work-up by extraction and the lower risk of enzyme loss. Furthermore, a biphasic continuous reaction is cheaper since the cofactor remains in the reactive phase making a continuous feed of cofactor unnecessary.

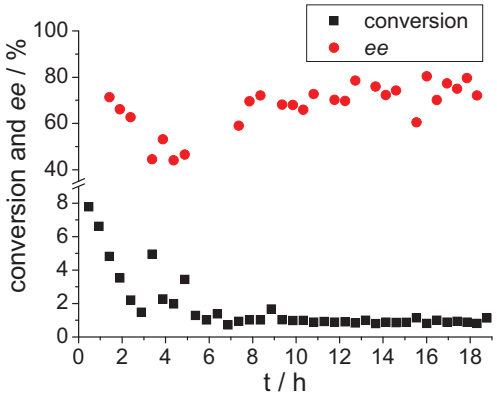


Figure 4-26 Continuous synthesis of (*S*)-2-butanol
(c (2-butanone) = 25 mmol L^{-1}, c (*L*-malic acid) = 60 mmol L^{-1}, c (NAD^+) = 0.1 mmol L^{-1}, c (SADH-X2) = 2.0 mg mL^{-1}, c (MDH) = 2.5 mg mL^{-1}, c (buffer) = 100 mmol L^{-1}, V (reaction) = 10.0 mL, flow rate = 0.45 ml min^{-1}, τ = 22.2 min, T = 30 °C, experiment: TH 08)

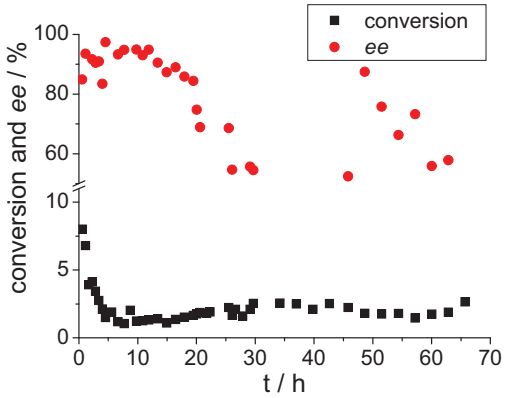


Figure 4-27 Continuous synthesis of (*S*)-2-butanol
(c (2-butanone) = 25 mmol L^{-1}, c (*L*-malic acid) = 60 mmol L^{-1}, c (NAD^+) = 0.1 mmol L^{-1}, c (SADH-X2) = 2.0 mg mL^{-1}, c (MDH) = 2.5 mg mL^{-1}, c (buffer) = 100 mmol L^{-1}, V (reaction) = 10.0 mL, flow rate = 0.36 ml min^{-1}, τ = 27.8 min, T = 30 °C, experiment: TH 09)

4.3 Summary of One-Phase Systems

The solid LB-ADH and the SADH-X2 were tested for the enantioselective reduction of 2-butanone to the respective corresponding alcohol in an aqueous one-phase system. The influence of the method of cofactor regeneration and the influence of reaction conditions were investigated. The reduction of 2-butanone using the SADH-X2 showed poor conversion of 11% when employing substrate dependent cofactor regeneration. At a 10-fold excess of 2-propanol racemisation was fast with 5.5% h^{-1} whereas it can be minimized to 0.65% h^{-1} by using a 100-fold excess of the co-substrate. If the same reactions were performed with enzyme dependent cofactor regeneration at different ADH:MDH ratios maximum conversion could be increased to 78%, but it decreased after passing through a maximum. Regarding the selectivity only the initial *ee* could be improved to maximum 92%, but racemisation was faster than with substrate dependent cofactor regeneration. The results show that the cofactor regeneration method has a strong influence on conversion and selectivity. For enzyme dependent cofactor regeneration a compromise in reaction conditions has to be found for the two-enzyme system.

Several experiments for the synthesis of (*R*)-2-butanol using the LB-ADH show that conversion and initial reaction rate are dependent on the 2-butanone and on the 2-propanol

concentration. By variation of these two parameters the reaction system could be optimized. Especially the initial reaction rate could be increased to 0.44 mmol L^{-1} min^{-1} by using high substrate and co-substrate concentrations. For the initial *ee* no dependence on either of the concentrations was observed. The racemisation rate decreased with increasing substrate concentration and could be lowered to 0.3% min^{-1} at a 2-propanol concentration of 2.0 mol L^{-1}. Up to now, the best conversion using the LB-ADH was obtained at 20 mmol L^{-1} 2-butanone and 0.2 mol L^{-1} 2-propanol concentration. Highest initial selectivity was found at the same reaction conditions.

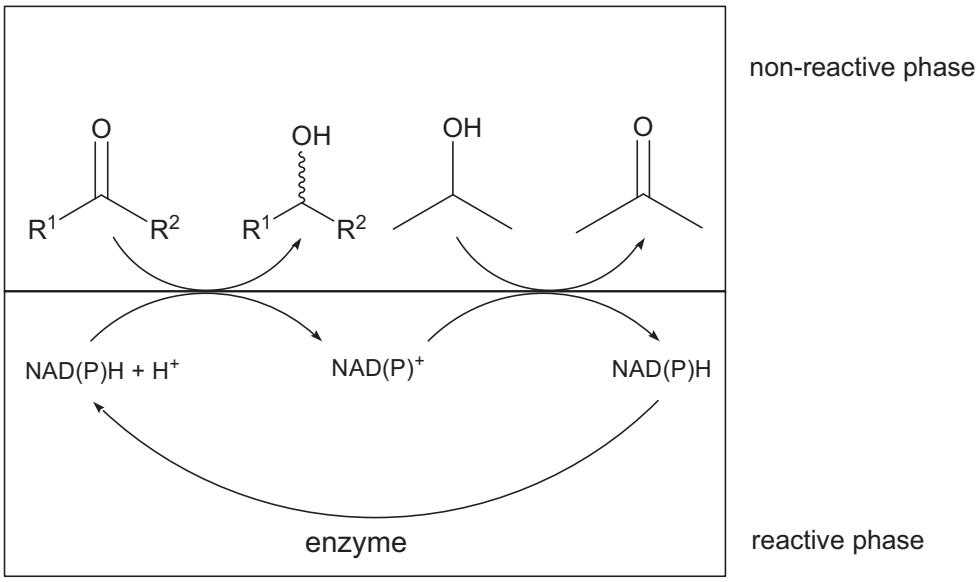
The racemisation mechanism of the LB-ADH and the SADH-X2 takes place via 2-butanone as intermediate (Scheme 4-1). The solid LB-ADH preparation showed high conversion of >99% and fast decrease in *ee* with (*R*)- and *rac*-2-butanol as substrate. Conversion to 2-butanone was even higher than the amount of $NADP^+$ allows for. In the absence of the oxidized cofactor conversion of (*R*)-2-butanol was still quantitative, and decrease in *ee* occured as well but was less distinctive. The treatment of the dissolved enzyme at 45 °C for 5 min resulted in quantitative conversion but suppressed decrease in *ee* to a minimum. The same experiments were conducted with a purified liquid LB-ADH preparation. Conversion and decrease in *ee* in presence of $NADP^+$ were less distinct. In absence of the oxidized cofactor no conversion was observed and *ee* did not change. The SADH-X2 converted (*S*)- and *rac*-2-butanol in presence of NAD^+ only in very small amounts and did not influence the *ee*. If no cofactor was present conversion was surprisingly higher.

In an enzyme membrane reactor it was possible to perform the continuous synthesis of (*S*)-2-butanol using the SADH-X2. The *ee* was positively influenced compared to batch reactions, but conversion only reached 2%. Enzyme could be washed out of the reactor which may be a reason for the low conversion, or the ADH may be very instable.

5 Synthesis of Chiral Alcohols using Two-Phase Systems

5.1 Determination of Partition Coefficients

For biocatalysis aqueous buffer is most often the straightforward choice as reactive phase. The non-reactive phase is formed by a water immiscible phase acting as reservoir for the substrate and as extraction phase for the product (Scheme 5-1). The system is dominated by substrate and product distribution between the reactive and the non-reactive phase. The partition coefficient P_X (Equation 5-1) of a single compound can be influenced by addition of a second compound, for example the co-substrate 2-propanol. Since 2-propanol is water-miscible it can act as a solubilizer and improve the solubility of the first compound in the reactive phase. In a common reaction system with substrate dependent cofactor regeneration four organic compounds are present: substrate, product, co-substrate, and co-product (Scheme 5-1). Since these four compounds influence each others partition coefficients this system is too complex to determine the partition. Therefore, P_X is determined only for single compounds. The C_4- to C_8-ketones and the aromatic model substrate acetophenone are used as well as the corresponding racemic alcohols. The optimum system would provide a low P_X for the ketone and a high P_X for the alcohol which would correspond to a high ketone and a low alcohol concentration in the reactive phase (Equation 5-1). As non-reactive phase MTBE and the tailor-made IL [PMIM][PF_6] are chosen. The respective phases are saturated with each other. A biphasic system with equal phase volumes containing one substrate is mixed thoroughly for 30 seconds and stored at 30 °C for 4 days. The given concentrations refer to the non-reactive phase. Samples are taken from both phases, and the concentrations of the compounds are determined by GC measurements using an internal standard. The partition coefficients are calculated using Equation 5-1.



Scheme 5-1 Two-phase reaction scheme with substrate dependent cofactor regeneration

$$P_X = \frac{c(\text{substrate})_{\text{non-reactive}}}{c(\text{substrate})_{\text{reactive}}}$$

Equation 5-1 Calculation of partition coefficient P_X

Ideally, partition coefficient will not be dependent on the ketone or alcohol concentration showing a constant correlation between P_X and concentration (Figure 5-1). Due to miscibility with either the reactive or the non-reactive phase, P_X will change with increasing concentration. In case of miscibility with the non-reactive phase partition coefficients will increase with increasing substrate concentration and will reach a limiting value (Figure 5-2). If the substrate is miscible with the reactive phase, P_X will decrease with increasing substrate concentration and reach a limiting value (Figure 5-3). Limiting values are defined by the miscibility limit.

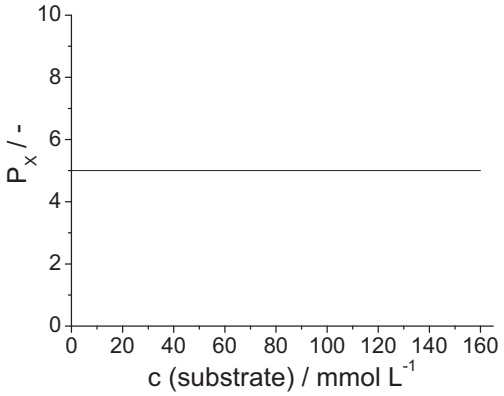


Figure 5-1 Illustration of ideal partition coefficients

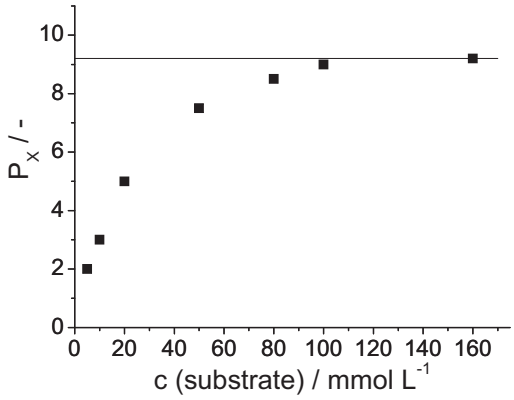


Figure 5-2 Illustration of non-ideal partition coefficients; substrate miscible with non-reactive phase

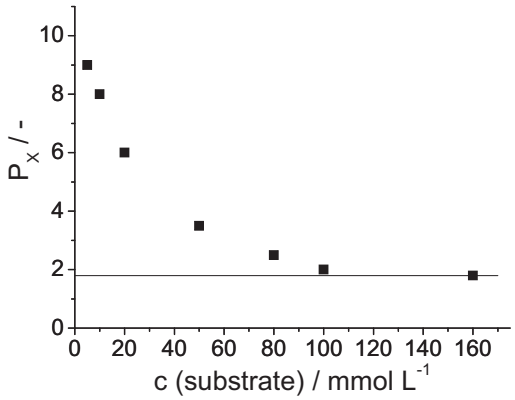


Figure 5-3 Illustration of non-ideal partition coefficients; substrate miscible with reactive phase

5.1.1 Partition Coefficients of Ketones

The partition coefficients of the investigated ketones in the systems H_2O/MTBE and H_2O/[PMIM][PF_6] mostly show to be dependent on the concentration of the respective substrate. They are non-ideal (Table 5-1). Furthermore, P_X is usually larger in the MTBE system revealing a better solubility of the ketones in MTBE than in the IL.

For 2-butanone the largest P_X of 6 is obtained in the MTBE system containing 0.2 mol L^{-1} 2-propanol (Figure 5-4). It decreases with increasing 2-butanone concentration displaying a rising solubility in the aqueous phase at higher substrate concentrations. It was expected that the presence of 2-propanol raises the solubility of the ketone in the aqueous phase resulting in a lower P_X. In the MTBE system without 2-propanol and in the IL system P_X increases with increasing substrate concentration (Figure 5-4) whereas P_X is larger in the MTBE system.

For acetophenone the largest P_X is found in the H_2O/[PMIM][PF_6] system (Figure 5-5). In the MTBE system P_X is non-ideal and dependent on the acetophenone concentration. The addition of 0.2 mol L^{-1} 2-propanol to the MTBE system causes a slight decrease in P_X. Due to the low solubility of acetophenone in water the partition coefficients are typically tenfold higher than for 2-butanone.

For 2-pentanone P_X is non-ideal in both the IL and the MTBE system (Figure 5-6). The H_2O/MTBE system offers higher P_X but the limiting P_X is not reached in the investigated range of concentration for both systems. Compared to 2-butanone the partition coefficients are higher since 2-pentanone is less soluble in water.

The determination of P_X for 2-hexanone shows non-ideal curves for both systems (Figure 5-7). Again, for the MTBE system the partition coefficients are higher than for the IL system. In both cases limiting values are not reached. With regard to water solubility P_X for 2-hexanone are higher than for 2-pentanone.

The increase in P_X with increasing chain length does not apply to 2-heptanone (Figure 5-8). Here, especially for low substrate concentrations the partition coefficients are lower than for 2-hexanone (Figure 5-7). The typical non-ideal behaviour can be observed again, and P_X has higher values in the MTBE than in the IL system. A limiting P_X for the IL system can be found at about 160 mmol L^{-1} 2-heptanone, whereas for the MTBE system P_X still rises with substrate concentration.

For 2-octanone P_X is lower than for 2-heptanone especially in the H_2O/IL system (Figure 5-9). For both systems the partition coefficients are non-ideal. Again, for the MTBE system P_X is higher than for the IL system.

Table 5-1 Overview over partition coefficients

	non-reactive phase		
ketone	MTBE	[PMIM][PF_6]	P_X is larger in
2-butanone	non-ideal	non-ideal	MTBE
acetophenone	non-ideal	non-ideal	[PMIM][PF_6]
2-pentanone	non-ideal	non-ideal	MTBE
2-hexanone	non-ideal	non-ideal	MTBE
2-heptanone	non-ideal	non-ideal	MTBE
2-octanone	non-ideal	non-ideal	MTBE

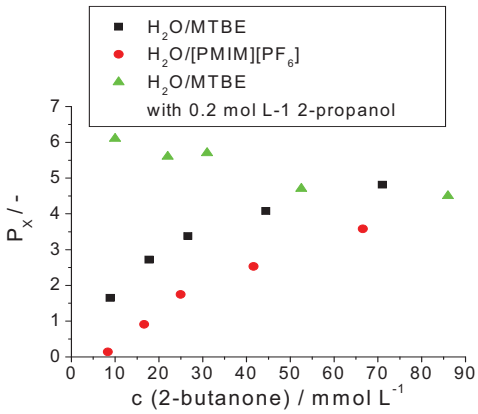


Figure 5-4 Partition coefficients of 2-butanone

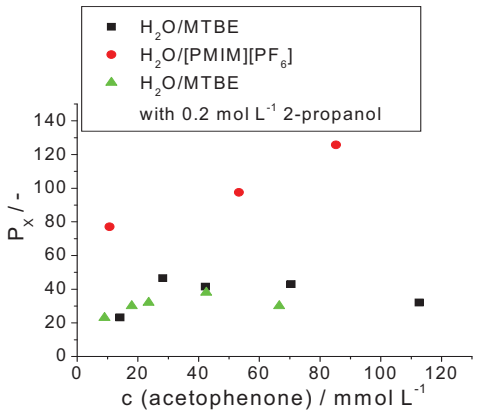


Figure 5-5 Partition coefficients of acetophenone

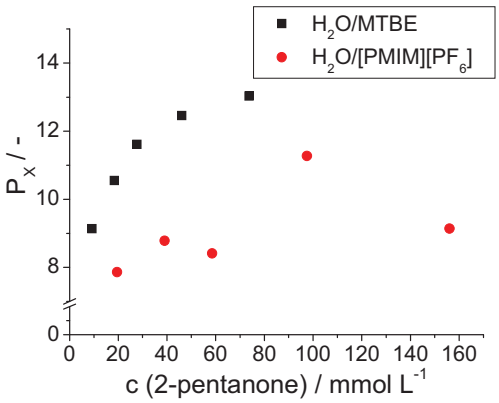


Figure 5-6 Partition coefficients of 2-pentanone

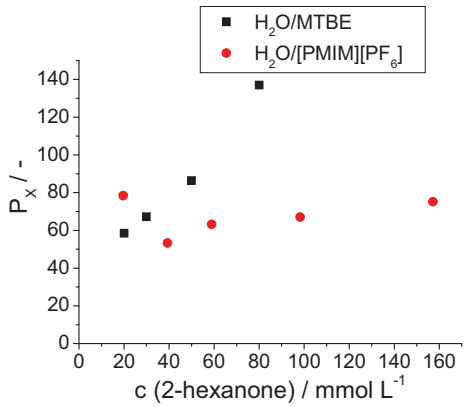


Figure 5-7 Partition coefficients of 2-hexanone

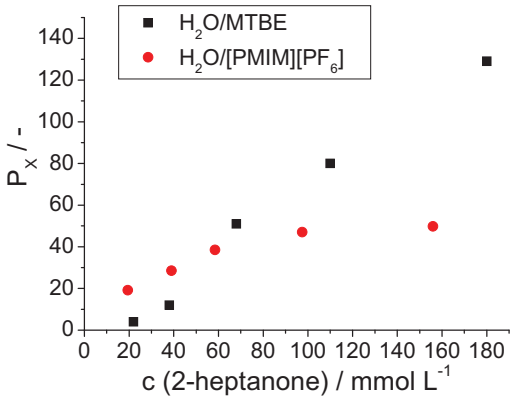


Figure 5-8 Partition coefficients of 2-heptanone

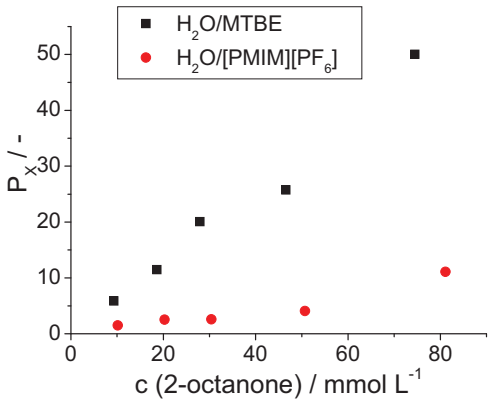


Figure 5-9 Partition coefficients of 2-octanone

5.1.2 Partition Coefficients of Alcohols

The partition coefficients of the investigated alcohols are of high interest in the H_2O/MTBE system. All show larger values for P_X than the H_2O/IL system and are generally non-ideal.
The partition coefficients for 2-butanol are non-ideal and do not reach a limiting value in all three investigated systems (Figure 5-10). The largest values are reached for the MTBE system. The addition of 0.2 mol L^{-1} of the co-substrate causes a decrease in P_X to values lower than those obtained for the IL system. In contrast to 2-butanone the presence of MTBE leads to a better solubility of the racemic alcohol in the aqueous phase and, therefore, to a lower P_X. The partition coefficients of *rac*-1-phenylethanol show the highest values in the MTBE system (Figure 5-11). The addition of 0.2 mol L^{-1} 2-propanol leads to a decrease in P_X. The lowest values for P_X are obtained for the H_2O/IL system. In contrast to acetophenone for *rac*-1-phenylethanol the partition coefficients are ideal for the MTBE system containing 2-propanol.
In the H_2O/MTBE system P_X is non-ideal for 2-pentanol (Figure 5-12). This behavior is also found for 2-pentanone (Table 5-1). In the IL system P_X is non-ideal and does not reach a limiting value in the investigated concentration range. For concentrations >150 mmol L^{-1} P_X does not exceed the values obtained for the MTBE system. Due to the lower water solubility of 2-pentanol compared to 2-butanol P_X is higher for the C_5-alcohol.
For 2-hexanol P_X is non-ideal in both the MTBE system and the IL system (Figure 5-13, Table 5-2). Again, the values are higher in the MTBE system. In the H_2O/MTBE system P_X is in the same range as for *rac*-2-pentanol, but in the IL system it is much lower and reaches a limiting value of about 2.5 at concentrations >160 mmol L^{-1}.
The partition coefficients of 2-heptanol are non-ideal in both systems (Figure 5-14, Table 5-2). P_X is larger in the MTBE system and reaches values >200 without converging. For the IL system the values settle in the range <10. The measured P_X for the racemic 2-heptanol are larger than those for 2-hexanol.
As already found for the partition of 2-octanone and 2-heptanone (Figure 5-9 and Figure 5-8) the partition coefficients of 2-octanol (Figure 5-15) are lower than those for 2-heptanol. P_X is higher in the MTBE system, and in both systems the partition coefficients are non-ideal. In the IL system they are much lower and converge towards a value around 11. In contrast, in the H_2O/MTBE system, they do not converge. Compared to the values measured for the corresponding ketone those for the alcohol are lower.

Table 5-2 Overview of partition coefficients of alcohols

	non-reactive phase		
alcohol	MTBE	[PMIM][PF_6]	P_X is larger in
rac-2-butanol	non-ideal	non-ideal	MTBE
rac-1-phenylethanol	non-ideal	non-ideal	MTBE
rac-2-pentanol	non-ideal	non-ideal	MTBE
rac-2-hexanol	non-ideal	non-ideal	MTBE
rac-2-heptanol	non-ideal	non-ideal	MTBE
rac-2-octanol	non-ideal	non-ideal	MTBE

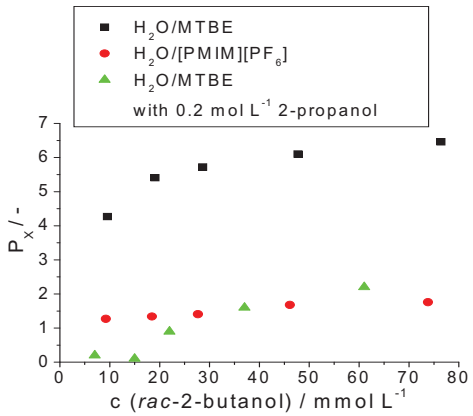


Figure 5-10 Partition coefficients of 2-butanol

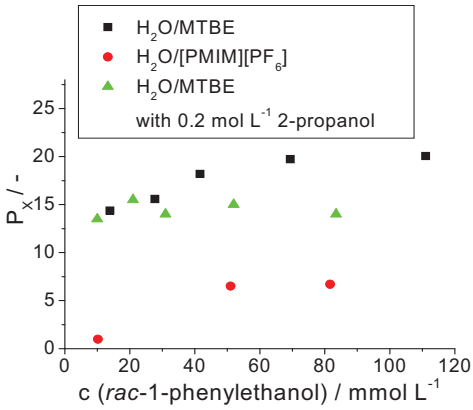


Figure 5-11 Partition coefficients of 1-phenylethanol

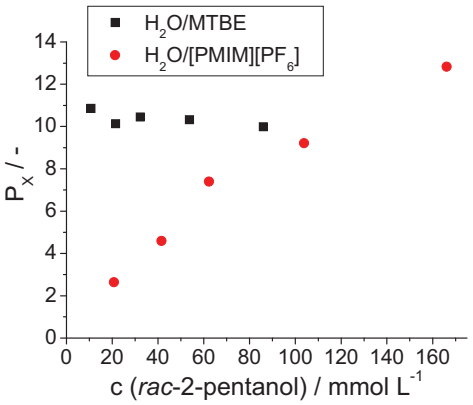


Figure 5-12 Partition coefficients of 2-pentanol

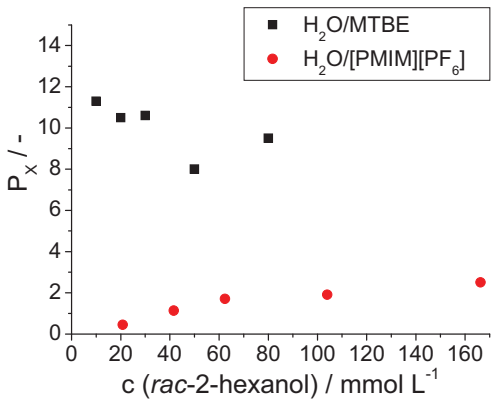


Figure 5-13 Partition coefficients of 2-hexanol

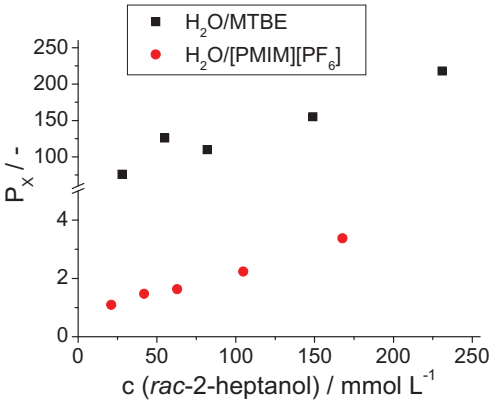


Figure 5-14 Partition coefficients of 2-heptanol

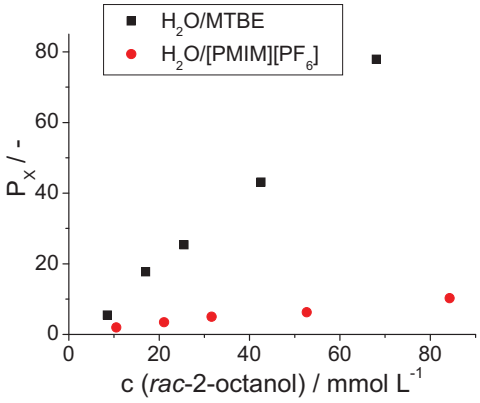


Figure 5-15 Partition coefficients of 2-octanol

5.2 Batch Reactions

5.2.1 Batch Synthesis of 2-Butanol with the System Buffer/MTBE

5.2.1.1 Substrate-dependent Cofactor Regeneration

The reduction of 2-butanone in a one-phase system using the LB-ADH shows low conversion and fast racemisation of the desired enantiopure (*R*)-alcohol (Chapter 4.1.3). Therefore, experiments are carried out using the two-phase system buffer/MTBE at the same reaction conditions as in the one-phase system (Table 5-3, for detailed graphs see Chapter 7.9.1). The aqueous buffer containing the 2-propanol and the cofactor is covered with equal volume MTBE containing the substrate. The reaction is started by addition of enzyme to the reactive phase.

The initial reaction rate shows dependence on the 2-butanone concentration as well as on the 2-propanol concentration (Figure 5-16, Table 5-4). With increasing 2-butanone concentration the initial reaction rate increases significantly according to hyperbolic adsorption kinetics (Michaelis-Menten kinetics). A tenfold increase in 2-propanol concentration causes an up to 2.6-fold increase in initial reaction rate (Figure 5-16). This is due to a faster regeneration of the cofactor, and, therefore, a better supply of the enzyme with reduced cofactor. For all except one experiment conversion reaches its equilibrium (Table 5-4). Comparing the percentaged conversion at constant 2-propanol concentration shows that conversion does not follow a clear trend but seems to decrease with increasing substrate concentration. The yield shows a clear increase with higher 2-butanone concentration for both 2-propanol concentrations. A higher co-substrate concentration has

the same effect. With one exception conversion and yield are higher at higher 2-propanol concentrations.

Enantiomeric excess and racemisation rate of the experiments (Figure 5-17, Table 5-5) do not show a clear tendency depending on the concentrations of substrate and co-substrate. Concerning the 2-propanol concentration racemisation rate is lower at higher concentrations. In presence of a very high excess of co-substrate the re-oxidation is suppressed. For all conducted experiments the initial enantiomeric excess is >99% and decreases over reaction time and with increasing conversion. Enantiomeric excess even decreases further when equilibrium conversion is reached. It would have been expected that the racemisation rate is lower at higher 2-butanone concentrations as the enzyme is saturated with substrate molecules, and, therefore, cannot re-oxidize the product alcohol. On the other hand it might be possible that due to higher product concentrations and related higher substrate concentrations racemisation is faster. The experimental results do not fit to either of these two approaches. The two-phase reaction system offers an access to improved conversion and selectivity compared to the one-phase system.

Table 5-3 Overview over experiments for the synthesis of (*R*)-2-butanol in the biphasic system buffer/MTBE using the LB-ADH

c (2-butanone) / mmol L^{-1}	c (2-propanol) / mol L^{-1}	
	0.2	2.0
20	BB 09	BB 10
50	BB 03	BB 05
100	BB 11	BB 12

Table 5-4 Conversion and initial reaction rates of the batch synthesis of (*R*)-2-butanol in the system buffer/MTBE using the LB-ADH

c (2-butanone) / mmol L^{-1}	max conversion / %		initial reaction rate / mmol L^{-1} min^{-1}		yield / mmol L^{-1}	
	c (2-propanol) / mol L^{-1}		c (2-propanol) / mol L^{-1}		c (2-propanol) / mol L^{-1}	
	0.2	2.0	0.2	2.0	0.2	2.0
20	40.6	36.9	0.151	0.121	8.1	7.4
50	39.7	65.3	0.203	0.524	14.9	32.7
100	30.4	41.1	0.403	0.641	30.4	41.1

Table 5-5 *ee* and racemisation rates of batch synthesis of (*R*)-2-butanol in the system buffer/MTBE using the LB-ADH

c (2-butanone) / mmol L^{-1}	max *ee* / %		racemisation rate / % min^{-1}	
	c (2-propanol) / mol L^{-1}		c (2-propanol) / mol L^{-1}	
	0.2	2.0	0.2	2.0
20	>99.0	>99.0	0.20	-
50	>99.0	>99.0	0.05	0.04
100	>99.0	>99.0	0.20	0.02

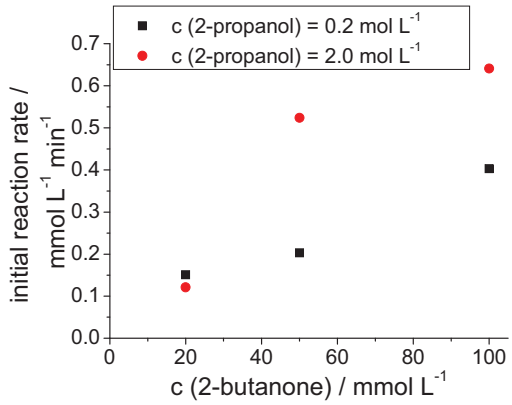


Figure 5-16 Initial reaction rates of the batch synthesis of (R)-2-butanol in the system buffer/MTBE using the LB-ADH

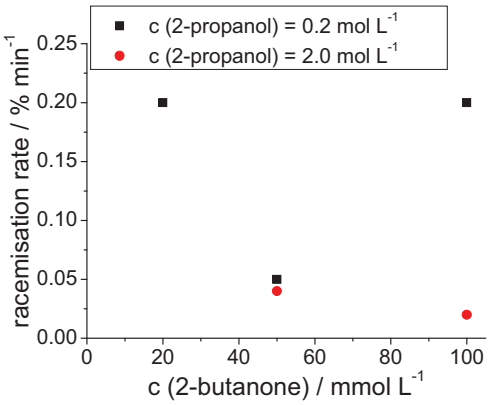


Figure 5-17 Racemisation rates of batch synthesis of (*R*)-2-butanol in the system buffer/MTBE using the LB-ADH

The liquid LB-ADH is characterized for the reduction of 2-butanone to (*R*)-2-butanol depending on the concentration of the ADH and of 2-propanol (Table 5-6). The initial reaction rate shows a linear correlation on the enzyme concentration (Figure 5-18, Table 5-7). The higher the enzyme concentration, the higher is the initial reaction rate. Up to 10 μL mL^{-1} LB-ADH concentration is linearly correlated with reaction rate. With higher concentrations of LB-ADH deviation from the linear behavior is observed. A possible explanation would be the agglomeration of the LB-ADH. 2-propanol concentration also has an effect on the initial reaction rate and on conversion. At 0.2 mol L^{-1} both reaction rate and conversion are lower than at 2.0 mol L^{-1} co-substrate. This, again, shows that at high 2-propanol concentrations the cofactor regeneration is faster which enables a faster reduction of the ketone. Furthermore, equilibrium is shifted to product side leading to higher conversions.

The racemisation rate is dependent on the LB-ADH concentration at 0.2 mol L^{-1} 2-propanol (Figure 5-19, Table 5-8). It strongly increases with increasing enzyme concentration. In contrast, a 10-fold co-substrate concentration of 2.0 mol L^{-1} suppresses the negative effect of high enzyme concentrations on the enantioselectivity (Figure 5-19, Table 5-8). For all three

tested LB-ADH concentrations the racemisation rate is 0.02% min^{-1} whereas it is 2- to 13-fold higher in presence of the lower co-substrate concentration. This is in line with the postulated mechanism that for the racemisation oxidized cofactor is necessary. For all reactions the initial *ee* is >99%.

Table 5-6 Overview over experiments for the synthesis of (*R*)-2-butanol in the biphasic system buffer/MTBE using the liquid LB-ADH

c (LB-ADH) / $\mu L\ mL^{-1}$	c (2-propanol) / $mol\ L^{-1}$	
	0.2	2.0
5	BB 18	BB 23
10	BB 19	BB 24
20	BB 04	BB 06

Table 5-7 Conversion and initial reaction rates of the batch synthesis of (*R*)-2-butanol in the system buffer/MTBE using the liquid LB-ADH

c (LB-ADH) / $\mu L\ mL^{-1}$	max conversion / % c (2-propanol) / $mol\ L^{-1}$		initial reaction rate / $mmol\ L^{-1}\ min^{-1}$ c (2-propanol) / $mol\ L^{-1}$		yield / $mmol\ L^{-1}\ min^{-1}$ c (2-propanol) / $mol\ L^{-1}$	
	0.2	2.0	0.2	2.0	0.2	2.0
5	20.6	30.3	0.091	0.129	10.3	15.2
10	29.7	39.9	0.180	0.248	14.9	20.0
20	30.9	29.2	0.531	0.220	15.5	14.6

Table 5-8 *ee* and racemisation rates of batch synthesis of (*R*)-2-butanol in the system buffer/MTBE using the liquid LB-ADH

c (LB-ADH) / $\mu L\ mL^{-1}$	max *ee* / % c (2-propanol) / $mol\ L^{-1}$		racemisation rate / % min^{-1} c (2-propanol) / $mol\ L^{-1}$	
	0.2	2.0	0.2	2.0
5	>99.0	>99.0	0.04	0.02
10	>99.0	>99.0	0.07	0.02
20	>99.0	>99.0	0.26	0.02

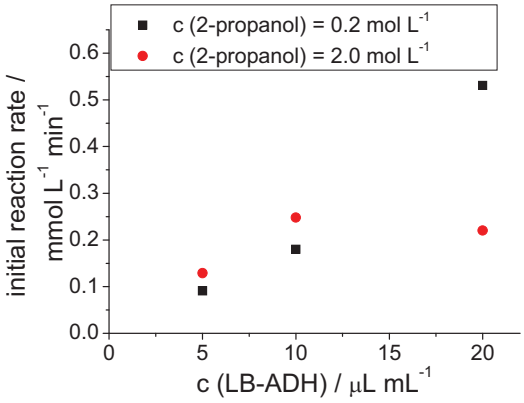


Figure 5-18 Initial reaction rates of biphasic batch reactions in the system buffer/MTBE using the liquid LB-ADH to synthesize (*R*)-2-butanol

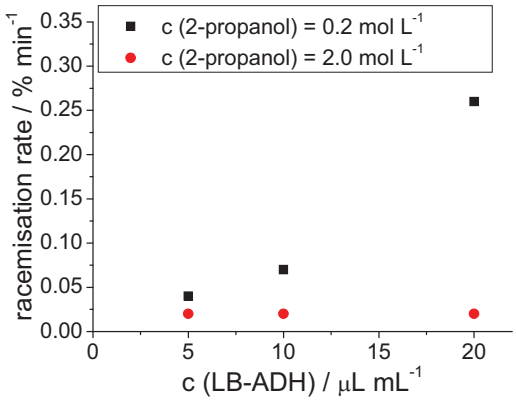
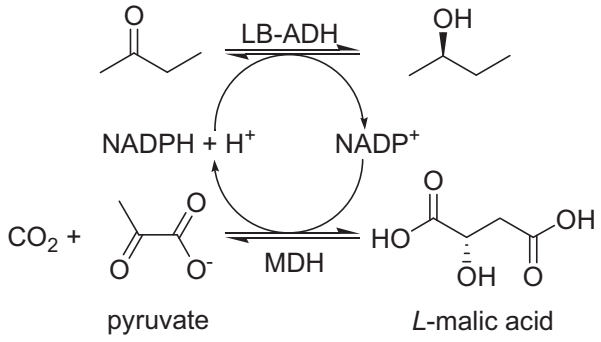


Figure 5-19 Racemisation rates of biphasic batch reactions in the system buffer/MTBE using the liquid LB-ADH to synthesize (*R*)-2-butanol

5.2.1.2 Enzyme-dependent Cofactor Regeneration

In the biphasic reaction system buffer/MTBE the reduction of 2-butanone to (*R*)-2-butanol is conducted employing enzyme dependent cofactor regeneration (Scheme 5-2). The reaction system is a multi-parameter system and complex to quantify under continuous reaction conditions. Therefore, it is important to conduct batch experiments to investigate the known issues:

- Due to fast cofactor regeneration a high NADPH concentration may occur and inhibit both the MDH and the LB-ADH.
- The high $NADP^+$ concentration caused by fast reduction of the ketone may inhibit both enzymes.
- The MDH may inhibit itself or the LB-ADH.
- High product alcohol concentrations may inhibit both enzymes.
- Pyruvate as co-product of the cofactor regeneration may inhibit both enzymes.



Scheme 5-2 Reduction of 2-butanone employing the LB-ADH and enzyme dependent cofactor regeneration utilizing the NADP-dependent MDH

The NADP-dependent MDH is applied at different concentrations between 1.0 and 12.0 mg mL^{-1} at 50 mmol L^{-1} 2-butanone and at 60 mmol L^{-1} *L*-malic acid concentration (Table 5-9). The initial reaction rate is strongly influenced by the MDH concentration (Figure 5-20, Table 5-10). Up to a MDH concentration of 8.0 mg mL^{-1} the reaction rate increases linearly to a maximum value of 0.37 mmol L^{-1} min^{-1} and decreases linearly at concentrations higher than 8.0 mg mL^{-1} MDH. An increase in MDH concentration enables faster cofactor regeneration, and, therefore, allows for a faster reduction of the ketone. The increase in initial reaction rate and conversion reverts if the MDH concentration reaches a certain value.

At the optimum MDH concentration of 8.0 mg mL^{-1} the reaction is also carried out at 20 mmol L^{-1} and 100 mmol L^{-1} 2-butanone concentration (Figure 5-21, Table 5-10). The initial reaction rate shows its highest value of 0.37 mmol L^{-1} min^{-1} at 50 mmol L^{-1} and is by a factor of 1.4 lower at 20 mmol L^{-1} and by a factor of 4.6 lower at 100 mmol L^{-1} (Table 5-10). The same trend applies for the conversion reached within the observed reaction time.

Racemisation rate as function of the MDH concentration does not show a maximum but increases linearly with increasing enzyme concentration (Figure 5-22, Table 5-11). This is counterintuitive and is not mirrored by the trends found for conversion and initial reaction rate. Racemisation starts directly at the beginning of the reaction and is linear. With increasing MDH concentration the initial enantiomeric excess increases (Figure 5-23, Table 5-11). This behavior is contrary to the increasing racemisation rate with increasing MDH concentration. One possible explanation is the very good supply with NADPH at the beginning of the reaction suppressing the re-oxidation of the product by allowing for fast conversion of the ketone. But since higher amounts of (*R*)-2-butanol are present at high MDH concentrations racemisation is faster. This explains the development of the racemisation rates. The 2-butanone concentration also has an effect on racemisation rate (Figure 5-24, Table 5-11). At constant MDH concentration of 8.0 mg mL^{-1} it decreases exponentially with increasing substrate concentration. Higher 2-butanone concentrations shift equilibrium to product side and inhibit product oxidation by occupying the LB-ADH with substrate.

Table 5-9 Overview over experiments for the synthesis of (*R*)-2-butanol in the biphasic reaction system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration

c (2-butanone) / mmol L^{-1}	c (MDH) / mg mL^{-1}	experiment
50	1	BB 13
50	2	BB 14
50	4	BB 15
50	6	BB 20
20	8	BB 26
50	8	BB 16
100	8	BB 25
50	10	BB 17
50	12	BB 21

Table 5-10 Conversion and initial reaction rates of the synthesis of (*R*)-2-butanol in the biphasic reaction system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration

c (2-butanone) / mmol L^{-1}	c (MDH) / mg mL^{-1}	max conversion / %	initial reaction rate / mmol L^{-1} min^{-1}
50	1	5.1	0.026
50	2	16.7	0.080
50	4	32.6	0.151
50	6	39.9	0.265
20	8	35.4	0.265
50	8	41.1	0.371
100	8	25.4	0.081
50	10	38.4	0.329
50	12	31.8	0.248

Table 5-11 *ee* and racemisation rates of the synthesis of (*R*)-2-butanol in the biphasic reaction system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration

c (2-butanone) / mmol L^{-1}	c (MDH) / mg mL^{-1}	max *ee* / %	racemisation rate / % min^{-1}
50	1	41.8	0.13
50	2	55.6	0.06
50	4	75.9	0.11
50	6	81.9	0.20
20	8	80.0	0.28
50	8	86.2	0.17
100	8	80.7	0.15
50	10	87.4	0.20
50	12	90.9	0.25

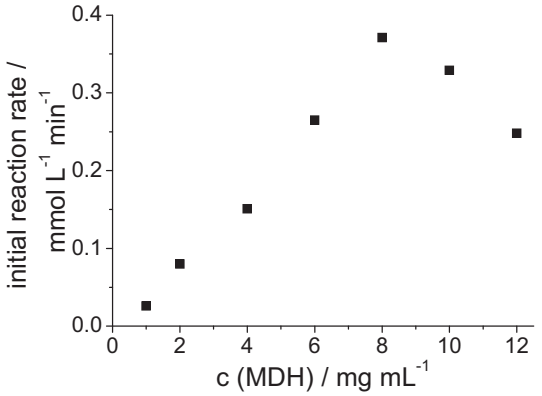


Figure 5-20 Initial reaction rates of biphasic batch reactions in the system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration to synthesize (*R*)-2-butanol (dependence on the MDH concentration)

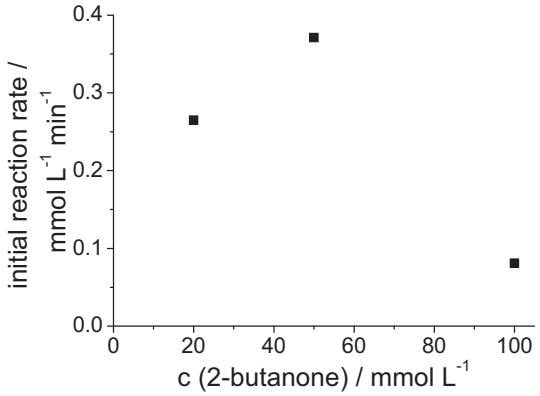


Figure 5-21 Initial reaction rates of biphasic batch reactions in the system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration to synthesize (*R*)-2-butanol (dependence on the 2-butanone concentration)

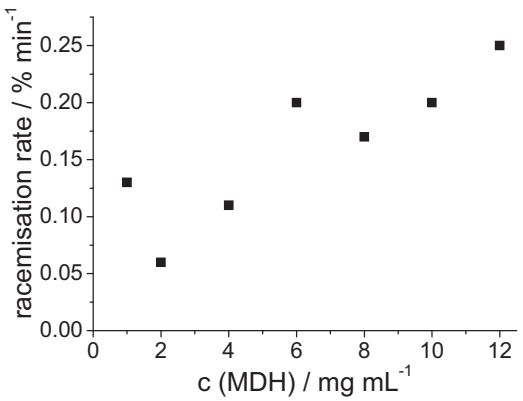


Figure 5-22 Racemisation rates of biphasic batch reactions in the system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration to synthesize (*R*)-2-butanol (dependence on the MDH concentration)

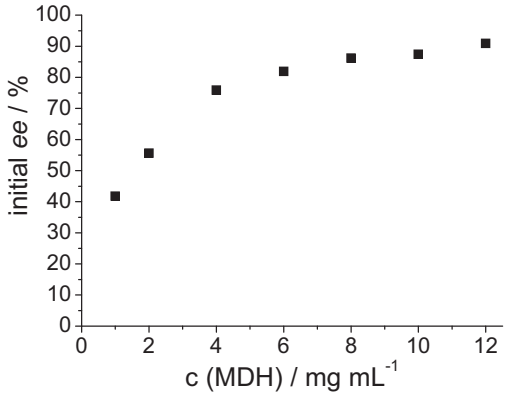


Figure 5-23 Initial *ee* of biphasic batch reactions in the system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration to synthesize (*R*)-2-butanol (dependence on the MDH concentration)

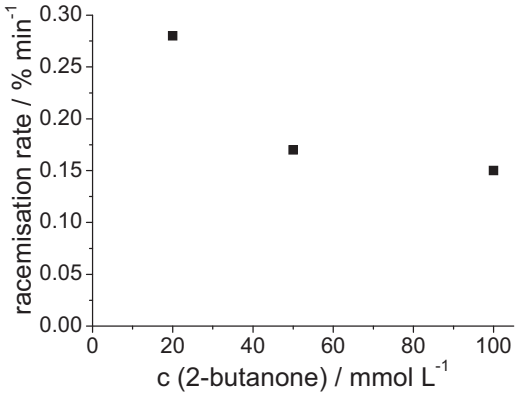


Figure 5-24 Racemisation rates of biphasic batch reactions in the system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration to synthesize (R)-2-butanol (dependence on 2-butanone concentration)

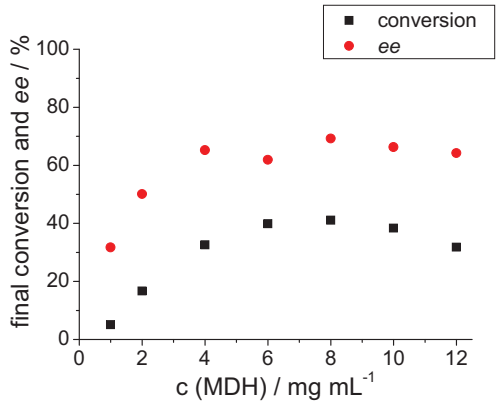


Figure 5-25 Comparison of final conversion and *ee* of biphasic batch reactions in the system buffer/MTBE using the LB-ADH and enzyme dependent cofactor regeneration to synthesize (*R*)-2-butanol (dependence on the MDH concentration)

5.2.2 Batch Synthesis of 2-Butanol in the System Buffer/[PMIM][PF_6]

The reduction of 2-butanone to (*R*)-2-butanol using the solid LB-ADH is carried out in a two-phase reaction system employing the tailor-made ionic liquid [PMIM][PF_6] as non-reactive phase (Table 5-12). The IL selectively extracts acetone out of the reactive phase but is immiscible with 2-propanol. This way, equilibrium of the cofactor regeneration is shifted. The initial reaction rate shows a maximum at 50 mmol L^{-1} 2-butanone concentration for both 0.2 and 2.0 mol L^{-1} 2-propanol (Figure 5-26, Table 5-13). A reason for the maximum in initial reaction rate may be that at higher substrate concentrations the product alcohol concentration is higher which causes a kinetically favored re-oxidation of the product, and, therefore, a decrease in reaction rate. High 2-propanol concentrations allow for higher initial reaction rates (Table 5-13). At high 2-propanol concentrations the rate of the cofactor regeneration increases which leads to a faster conversion of 2-butanone compared to lower co-substrate concentrations.

The initial enantiomeric excess is >99% for all reactions, but at a 2-propanol concentration of 0.2 mol L^{-1} it decreases with time (Table 5-14). Racemisation rate has a maximum at 50 mmol L^{-1} 2-butanone. At 2.0 mol L^{-1} co-substrate concentration racemisation is only observable at 50 mmol L^{-1} 2-butanone. It is by a factor of 10 lower than at 0.2 mol L^{-1} 2-propanol. Obviously, the racemisation is connected to the initial reaction rate which also

showed maximum values at 50 mmol L^{-1} (Table 5-13). The faster the product is formed the faster it is racemized. High co-substrate concentrations effectively prevent racemisation.

Table 5-12 Overview over experiments for the synthesis of (*R*)-2-butanol in the biphasic system buffer/[PMIM][PF_6] using the solid LB-ADH

c (2-butanone) / mmol L^{-1}	c (2-propanol) / mol L^{-1}	
	0.2	2.0
20	PM 167	AL 20
50	AL 12	AL 10
100	AL 19	AL 18

Table 5-13 Conversion and initial reaction rates of the synthesis of (*R*)-2-butanol in the biphasic reaction system buffer/[PMIM][PF_6] using the solid LB-ADH

c (2-butanone) / mmol L^{-1}	max conversion / %		initial reaction rate / mmol L^{-1} min^{-1}		product concentration / mmol L^{-1}	
	c (2-propanol) / mol L^{-1}		c (2-propanol) / mol L^{-1}		c (2-propanol) / mol L^{-1}	
	0.2	2.0	0.2	2.0	0.2	2.0
20	12.6	14.0	0.025	0.108	2.5	2.8
50	7.4	14.6	0.085	0.225	3.7	7.3
100	4.3	8.0	0.040	0.085	4.3	8.0

Table 5-14 *ee* and racemisation rates of the synthesis of (*R*)-2-butanol in the biphasic reaction system buffer/[PMIM][PF_6] using the solid LB-ADH

c (2-butanone) / mmol L^{-1}	max *ee* / %		racemisation rate / % min^{-1}	
	c (2-propanol) / mol L^{-1}		c (2-propanol) / mol L^{-1}	
	0.2	2.0	0.2	2.0
20	>99.0	>99.0	0.03	-
50	>99.0	>99.0	0.21	0.02
100	>99.0	>99.0	0.01	-

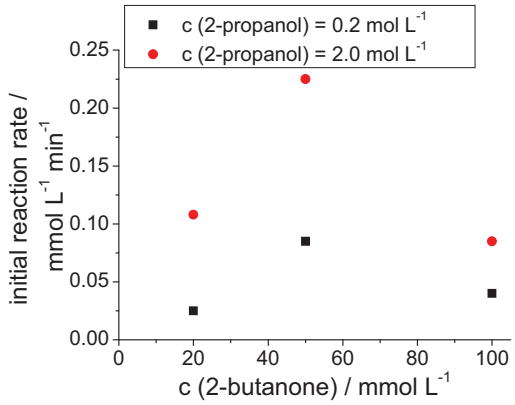


Figure 5-26 Initial reaction rates of biphasic batch reactions in the system buffer/[PMIM][PF_6] using the LB-ADH to synthesize (*R*)-2-butanol

5.2.3 Batch Synthesis of (*R*)-1-Phenylethanol

The synthesis of (*R*)-1-phenylethanol is performed using the two-phase reaction systems buffer/MTBE and buffer/[PMIM][PF_6] employing the LB-ADH and substrate dependent cofactor regeneration (Figure 5-27). In both systems the reaction proceeds very slowly, in the IL system even slower than in the MTBE system. After 4 h conversion is 12% in the system buffer/MTBE whereas it is only 4% in the system containing the IL. The enantiomeric excess is >99%. The MTBE system is more suitable because within the same reaction time it reaches a three-fold conversion compared to the IL system. The determination of the partition coefficients already showed that acetophenone has a higher solubility in the IL than in MTBE (see Chapter 5.1.1) which causes lower concentrations in the aqueous phase and, therefore, lower conversion. In contrast, the product alcohol 1-phenylethanol is better soluble in MTBE (see Chapter 5.1.2) allowing for a better extraction out of the reactive phase. Therefore, the system buffer/MTBE will be employed for continuous synthesis of (*R*)-1-phenylethanol.

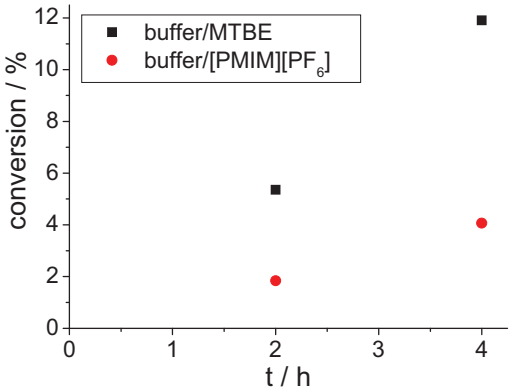


Figure 5-27 Batch synthesis of (*R*)-1-phenylethanol in the systems buffer/MTBE and buffer/[PMIM][PF_6] (c (acetophenone) = 20 mmol L^{-1}, c (2-propanol) = 0.4 mol L^{-1}, c ($NADP^+$) = 2.0 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiments: PM 131 and 132)

5.2.4 Batch Synthesis of (*R*)-2-Hexanol

Another substrate of interest is 2-hexanone. The batch synthesis of (*R*)-2-hexanol is carried out in the two-phase reaction system buffer/MTBE using the LB-ADH and substrate dependent cofactor regeneration. Especially the influence of the 2-propanol concentration on the initial reaction rate v_0 is investigated. A set of 24 experiments is performed with addition of substoichiometrical, stoichiometrical, and excess amounts of the co-substrate. Furthermore, the concentrations of 2-hexanone and of the LB-ADH are varied (Table 5-15 and Table 5-16). For the influence of the 2-hexanone concentration on the initial reaction rate no clear trend can be observed (Table 5-15). For example, at 0.2 mmol L^{-1} 2-propanol at a LB-ADH concentration of 1.0 mg mL^{-1} v_0 shows a maximum at 50 mmol L^{-1} 2-hexanone (experiments DM 01, 03, 05). In contrast, at 2.0 mmol L^{-1} co-substrate and 1.0 mg mL^{-1} LB-ADH v_0 is positively influenced by higher substrate concentrations (experiments DM 02, 04, 06). At 2.0 mmol L^{-1} 2-propanol concentration and 0.5 mg mL^{-1} LB-ADH v_0 has a minimum at 50 mmol L^{-1} 2-hexanone.

The dependence of v_0 on the 2-propanol concentration at constant 2-hexanone and constant LB-ADH concentration shows a clear trend (Table 5-16, Figure 5-28 to Figure 5-30). With increasing co-substrate concentration v_0 increases. Independent from the enzyme concentration the highest initial reaction rates are obtained at 50 mmol L^{-1} 2-hexanone. The results show that it is essential to use an excess of co-substrate to achieve sufficient reaction rates as well as high conversions. An excess of co-substrate is the driving force for the reaction. Furthermore, at substoichiometrical 2-propanol concentrations conversion is restricted to the maximum amount of co-substrate available. Regarding the *ee* of the reaction

no loss is found over the investigated reaction time, but it is possible that in presence of very low amounts of 2-propanol the product alcohol is oxidized for cofactor regeneration. Smaller chiral product alcohols may not only be oxidized but even racemized in presence of little co-substrate concentrations as previous experiments for the synthesis of (*R*)- and (*S*)-2-butanol already showed with excess amounts of 2-propanol.

Table 5-15 v_0 depending on the 2-hexanone concentration

experiment	c (2-hexanone) / mmol L^{-1}	c (2-propanol) / mmol L^{-1}	v_0 / mmol L^{-1} min^{-1}	c (LB-ADH) / mg mL^{-1}
DM 01	20	0.2	0.0006	1.0
DM 03	50	0.2	0.0016	1.0
DM 05	100	0.2	0.0009	1.0
DM 02	20	2.0	0.0039	1.0
DM 04	50	2.0	0.0041	1.0
DM 06	100	2.0	0.0056	1.0
DM 07	20	0.2	0.0008	0.5
DM 09	50	0.2	0.0010	0.5
DM 11	100	0.2	0.0009	0.5
DM 08	20	2.0	0.0082	0.5
DM 10	50	2.0	0.0031	0.5
DM 12	100	2.0	0.0047	0.5

Table 5-16 v_0 depending on the 2-propanol concentration

experiment	c (2-hexanone) / mmol L^{-1}	c (2-propanol) / mmol L^{-1}	v_0 / mmol L^{-1} min^{-1}	c (LB-ADH) / mg mL^{-1}
DM 01	20	0.2	0.0006	1.0
DM 02	20	2.0	0.0039	1.0
DM 13	20	20.0	0.0156	1.0
DM 16	20	200.0	0.0267	1.0
DM 03	50	0.2	0.0016	1.0
DM 04	50	2.0	0.0041	1.0
DM 14	50	50.0	0.0252	1.0
DM 17	50	500.0	0.0441	1.0
DM 05	100	0.2	0.0009	1.0
DM 06	100	2.0	0.0056	1.0
DM 15	100	100.0	0.0310	1.0
DM 18	100	1000.0	0.0340	1.0
DM 07	20	0.2	0.0008	0.5
DM 08	20	2.0	0.0082	0.5
DM 19	20	20.0	0.0119	0.5
DM 22	20	200.0	0.0101	0.5
DM 09	50	0.2	0.0010	0.5
DM 10	50	2.0	0.0031	0.5
DM 20	50	50.0	0.0181	0.5
DM 23	50	500.0	0.0212	0.5
DM 11	100	0.2	0.0009	0.5
DM 12	100	2.0	0.0047	0.5
DM 21	100	100.0	0.0177	0.5
DM 24	100	1000.0	0.0176	0.5

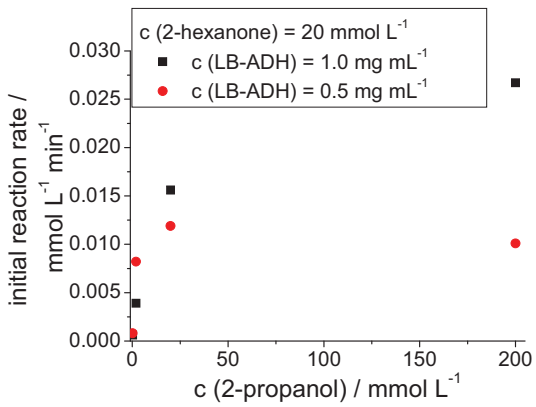


Figure 5-28 Initial reaction rate for the synthesis of (*R*)-2-hexanol in the biphasic reaction system buffer/MTBE (c (2-hexanone) = 20 mmol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, V (phase) = 5.0 mL, experiments: DM 01, 02, 07, 08, 13, 16, 19, 22)

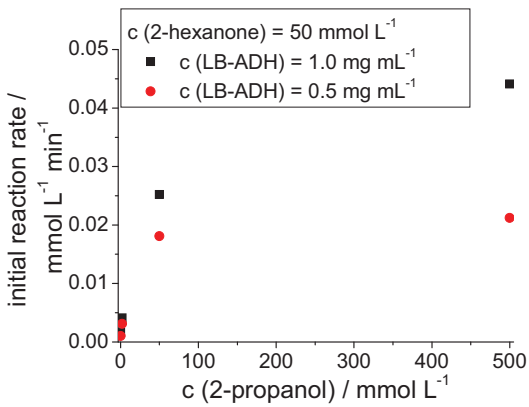


Figure 5-29 Initial reaction rate for the synthesis of (*R*)-2-hexanol in the biphasic reaction system buffer/MTBE (c (2-hexanone) = 50 mmol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (buffer) = 50 mmol L^{-1}, V (phase) = 5.0 mL, experiments: DM 03, 04, 09, 10, 14, 17, 20, 23)

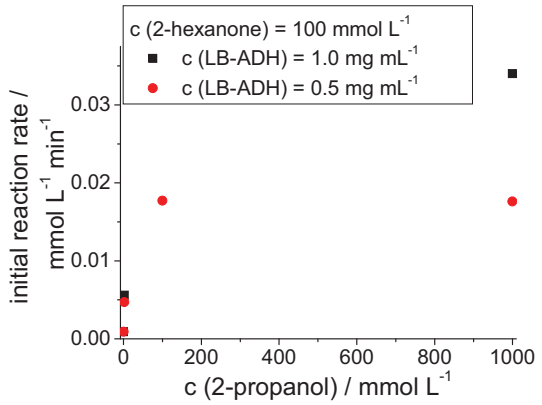


Figure 5-30 Comparison of the initial reaction rate for the synthesis of (*R*)-2-hexanol in the biphasic reaction system buffer/MTBE
(c (2-hexanone) = 100 mmol L^{-1}, c (NADP+) = 0.1 mmol L^{-1}, c (buffer) = 50 mmol L-1, V (phase) = 5.0 mL, experiments: DM 05, 06, 11, 12, 15, 18, 21, 24)

5.3 Continuous Reactions with the System Buffer/MTBE

5.3.1 Continuous Reaction Set-Up

A continuous reaction setup for a two-phase reaction system is used as follows: The substrate reservoir provides the feed substrate solution (non-reactive phase), i.e. 2-butanone and 2-propanol dissolved in MTBE, and maintains it at 4.0 °C. A syringe pump feeds the substrate solution into the non-reactive phase of the reactor while the directly coupled piston pump transfers the equal volume of this phase out of the reactor. The biphasic system is independently stirred from the top and bottom. The composition of each 5.0 mL phase is uniform. No emulsion is formed to enable online GC analysis. The reactor is held constant at 30 °C. Upon leaving the reactor, the non-reactive phase is pumped through the GC flow cell and into a waste bottle (Figure 5-31).[113] At regular time intervals, an autosampler takes samples from the flow cell and takes 1 μL of a standard 100 mmol L^{-1} 1-butanol solution before injecting the sample and standard together into the GC for analysis. The given concentrations and flow rates refer to the reactive or the non-reactive phase, respectively, i.e. to a volume of 5 mL.

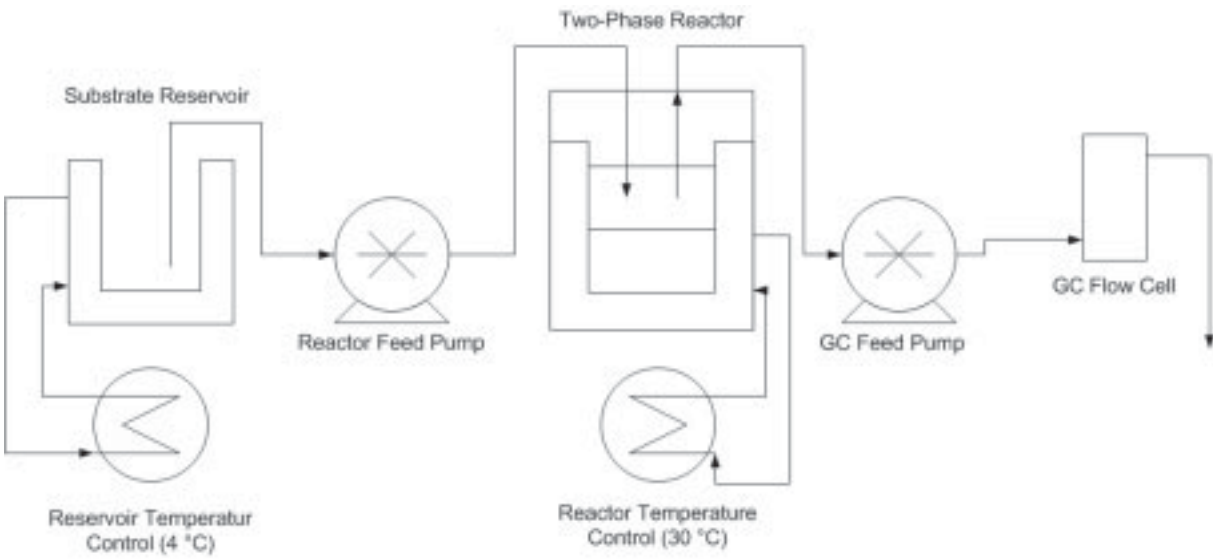


Figure 5-31 Flow scheme of continuous reaction set-up

5.3.2 Continuous Synthesis of (*R*)-1-Phenylethanol

As a model substrate acetophenone is continuously reduced to (*R*)-1-phenylethanol using the LB-ADH in the two-phase reaction system buffer/MTBE. The enzyme does not show any loss in activity over 138 h reaction time (Figure 5-32). The reaction is started at a flow rate of 40 μL min^{-1} which corresponds to a residence time τ of 125 min (Figure 5-32). The reaction reaches equilibrium after 10 h (4.8 τ), conversion in the steady state is between 8% and 9%. After 67 h (22.1 τ) the flow rate is lowered to 20 μL min^{-1} (τ = 250 min) (Figure 5-32). The system needs again 10 h which now corresponds to 2.4 τ to reach steady state. Conversion is constant at 12% for the next 58 h (13.9 τ). The enantiomeric excess is >99% over 138 h reaction time. With 8% to 12% conversion is low, but higher than in the batchwise experiments (Chapter 5.2.3). In the biphasic reaction system containing MTBE the LB-ADH shows to be exceptionally stable. The cofactor $NADP^+$ proves a very high stability as well. The TTN for the first steady state is 1094, for the second part of the reaction it is 835. These values are higher than expected since nicotine amide cofactors are known to be sensitive towards organic solvents like MTBE. The conversion of acetophenone by the LB-ADH is 0.11 mmol mg^{-1} and 0.08 mmol mg^{-1}, respectively. These values are low but promising and improvable, e.g. by optimizing reaction conditions.

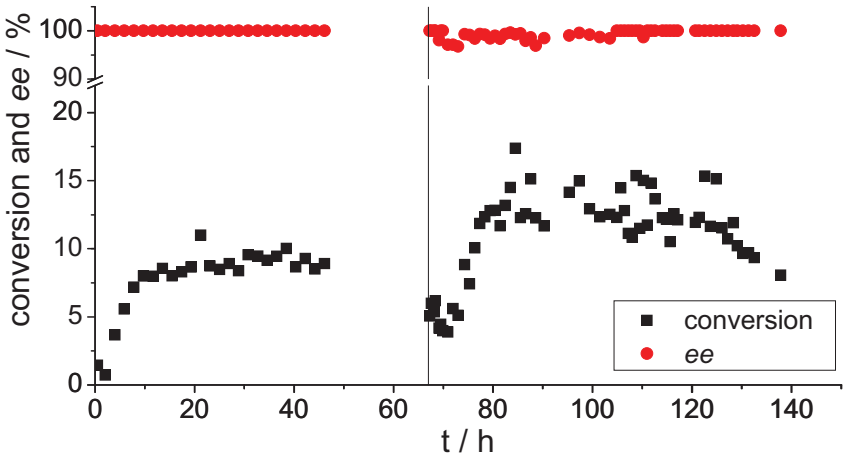


Figure 5-32 Continuous synthesis of (*R*)-1-phenylethanol in the systems buffer/MTBE (c (acetophenone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (phase) = 5.0 mL, flow rate = 40 μL min^{-1} (46 h), τ = 125 min, experiment: BB 38; flow rate = 40 μL min^{-1} 46-138 h), τ = 250 min, experiment: BB 39)

5.3.3 Continuous Synthesis of (*R*)-2-Butanol

The reduction of 2-butanone to (*R*)-2-butanol using the LB-ADH is conducted continuously in the system buffer/MTBE. The continuous experiments are done at different flow rates. Furthermore, the 2-butanone concentration is varied (Table 5-17). Starting at a flow rate of 42 μL min^{-1} (τ = 119 min) at an initial 2-butanone concentration of 50 mmol L^{-1} the reaction system needs 12 h (6.1 τ) to reach steady state with an equilibrium conversion of 70% (Figure 5-33). The steady state is maintained for 9 h (4.5 τ) without any loss in enzyme activity. The *ee* increases slightly within the first 3 h reaction time from 88.3% to 96.3% (4% h^{-1}) and decreases down to 90.5% in the steady state within the following 18 h (0.32% h^{-1}). Due to the continuous extraction of the product alcohol the decrease in *ee* is much lower compared to the corresponding batch experiment (Table 5-3, experiment BB 06). After 21 h the flow rate is increased to 60 μL min^{-1} (τ = 83.3 min). The reaction system responds to the shorter residence time within 1.5 h (1.1 τ) and reaches its new equilibrium conversion of about 52%. The *ee* increases slightly with a time delay of 9 h (6.5 τ) to 92.4% (0.2% h^{-1}). Within the following 14 h (10.1 τ) *ee* decreases to 89.3% (0.2% h^{-1}). The continuous experiment shows that shortening the residence time causes a decrease in conversion but a slight increase in *ee* since due to the shorter reaction time not only the reduction of the ketone is reduced but also racemisation.

A second continuous reaction is conducted at a flow rate of 50 μL min^{-1} (τ = 100 min) at an initial 2-butanone concentration of 50 mmol L^{-1} (Figure 5-34). Steady state is reached after

4.6 h (2.8 τ) with a conversion of 65%. Within the steady state of 15.7 h (9.4 τ) the LB-ADH does not show any loss in activity. The initial *ee* is 97% and decreases to 92.3% within the complete reaction time of 20.3 h (12.2 τ) which corresponds to a racemisation rate of 0.2% h^{-1}.

At an initial 2-butanone concentration of 100 mmol L^{-1} a third continuous reaction is started with a flow rate of 50 μL min^{-1} (τ = 100 min) to investigate the influence of the substrate concentration on the course of the reaction (Figure 5-35). The system does not reach a steady state within 46 h (27.6 τ). After 10 h the increase in conversion flattens, but it still increases to 45%. After 46 h the flow rate is changed to 40 μL min^{-1} (τ = 125 min). Within 14 h (6.7 τ) the system reacts to the change but, again, does not reach a steady state within 25 h (12 τ), but with 57% after 25 h conversion is higher than at the shorter residence time. After 71 h overall reaction the flow rate is lowered to 32 μL min^{-1} (τ = 156.3 min). The system needs 12 h (4.6 τ) to react to the change and seems to be in a steady state for the following 14 h. Conversion is in the same range as at τ = 125 min, and the LB-ADH does not lose any activity over the complete reaction time of 94 h. The initial *ee* is 94%, it continuously decreases at a rate of 0.1% h^{-1} to 85.5%. At the points where the flow rate is changed the *ee* slightly increases and then decreases further.

Compared to the continuous reduction of acetophenone conversion is higher, but it takes longer for the reaction system to respond to a change in flow rate. As expected, the *ee* for the reduction of 2-butanone is lower and decreases over reaction time. Compared to the batch experiments selectivity is up to 25% better due to the extraction of the product alcohol out of the reactive phase. The TTN for $NADP^+$ of up to 8208 and the productivity of the LB-ADH of up to 0.82 mmol mg^{-1} (Table 5-17) and the stability of the enzyme are promising. Together with the good enantiomeric excess and conversion they reveal the high potential of the investigated two-phase reaction system.

Table 5-17 Reaction conditions for the continuous reduction of 2-butanone

experiment	c (2-butanone) / mmol L^{-1}	flow rate / μL min^{-1}	τ / min	steady state	TTN ($NADP^+$)	Productivity (LB-ADH) / mmol mg^{-1}
BB 30	50	42	119	9 h, 4.5 τ	1588	0.16
	50	60	83.3	19 h, 13.7 τ	3557	0.42
BB 32	50	50	100	15.7 h, 9.4 τ	3062	0.31
BB 34	100	50	100	36 h, 216 τ	8208	0.82
	100	40	125	19 h, 9.1 τ	4469	0.44
	100	32	156.3	14 h, 5.4 τ	2700	0.28

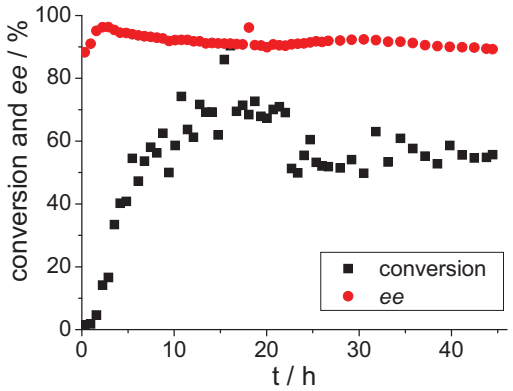


Figure 5-33 Continuous synthesis of (*R*)-2-butanol in the systems buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (phase) = 5.0 mL, flow rate = 42 µL min^{-1} (21 h, τ = 125 min), flow rate = 60 µL min^{-1} (21-44 h, τ = 83.3 min), experiment: BB 30)

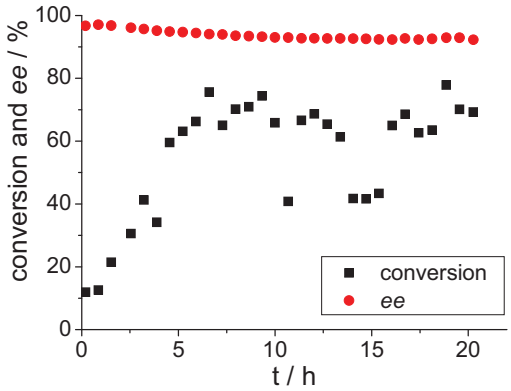


Figure 5-34 Continuous synthesis of (*R*)-2-butanol in the systems buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (phase) = 5.0 mL, flow rate = 50 µL min^{-1} (τ = 100 min), experiment: BB 32)

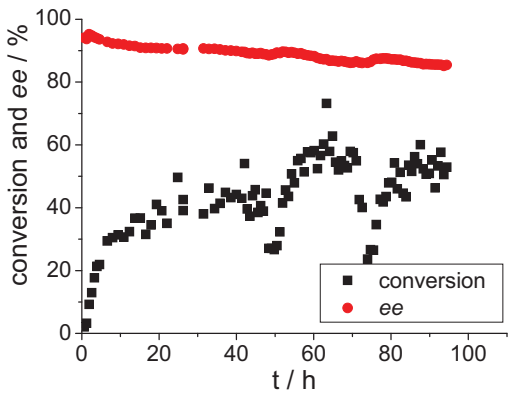


Figure 5-35 Continuous synthesis of (*R*)-2-butanol in the systems buffer/MTBE (c (2-butanone) = 100 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (phase) = 5.0 mL, flow rate = 50 µL min^{-1} (46 h, τ = 125 min), flow rate = 40 µL min^{-1} (46-71 h, τ = 125 min), flow rate = 32 µL min^{-1} (71-94 h, τ = 156.25 min), experiment: BB 34)

5.4 Summary of Two-Phase Systems

It has been shown that with appropriate choice of non-reactive phase and reaction conditions it is possible to utilize the advantages of a two-phase reaction system to overcome limitations

like substrate solubility, low conversion, and low enantioselectivity. As non-reactive phase MTBE and the IL [PMIM][PF_6] were chosen. First, the two systems buffer/MTBE and buffer/[PMIM][PF_6] were investigated regarding the partition coefficients of the prochiral ketones and the corresponding alcohols. They are non-ideal, i.e. dependent on the concentration of the respective compound. For the MTBE system partition coefficients of the ketones vary between 1 and 140, for the IL system the values are settled between 1 and 130, respectively depending on the ketone. The alcohols have P_X between 4 and 120 in the MTBE system and between 0.5 and 15 in the IL system. The ideal partition coefficients would be independent from the concentration, small for the ketone, and large for the alcohol. In the IL system the partition coefficients of the ketones are smaller compared to the MTBE system. This would make the IL system the better choice since it allows for higher ketone concentration in the reactive phase. The P_X for the alcohols have higher values in the MTBE system. Since it is important that the extraction of the alcohols out of the aqueous phase is good the MTBE system will be the preferred system for biphasic reactions.

In the biphasic reaction system buffer/MTBE the reduction of 2-butanone using the LB-ADH was conducted employing substrate dependent cofactor regeneration. In principal, an increase in 2-butanone and 2-propanol concentration influenced conversion and initial reaction rate positively. Initial *ee* and racemisation rate were able to be influenced by changes in substrate and co-substrate concentration as well, but an explicit trend was not observable. As expected, compared to the one-phase system conversion was higher and racemisation was less pronounced in the two-phase reaction system. The extraction of the product alcohol out of the reactive phase led to a shift in equilibrium and minimized product racemisation.

For the reactions done with the liquid LB-ADH preparation conversion and initial reaction rate increased with higher ADH and co-substrate concentration. At 0.2 mol L^{-1} 2-propanol concentration racemisation rate was increased by higher ADH concentrations, whereas at 2.0 mol L^{-1} co-substrate no dependence was observed.

Several experiments were conducted with enzyme dependent cofactor regeneration using the solid LB-ADH and the NADP-dependent MDH. Conversion and initial reaction rate were dependent on the MDH concentration and had a maximum at 8 mg mL^{-1}. Initial *ee* and racemisation rate increased with increasing MDH concentration, whereas the final *ee* had a maximum at 8 mg mL^{-1} MDH concentration. Depending on the 2-butanone concentration maximum conversion and *ee* had a maximum at 50 mmol L^{-1} while racemisation rate decreased with increasing substrate concentration. In principal, when using enzyme dependent cofactor regeneration conversion was lower, but also less racemisation occurred due to low product concentration.

For the buffer/[PMIM][PF_6] system a decrease in conversion at higher substrate concentrations was observed, whereas a higher co-substrate concentration had a positive effect on conversion. Initial reaction rate showed a maximum at 50 mmol L^{-1} 2-butanone and was positively influenced at higher 2-propanol concentration. At 0.2 mol L^{-1} racemisation rate had a maximum at 50 mmol L^{-1} substrate concentration, at 2.0 mol L^{-1} racemisation was only observable at 50 mmol L^{-1} 2-butanone. Compared to the MTBE system conversion was lower in the IL system, but also racemisation was lower.

For the synthesis of (*R*)-2-hexanol using the LB-ADH an increase of the initial reaction rate with higher 2-propanol concentration was observed. A higher LB-ADH concentration also had a positive effect on v_0. Depending on the 2-hexanone concentration no general dependence was determined. The 2-propanol concentration restricted the overall conversion and was needed in high excess to enable high conversion. The enantiomeric excess was >99% and was not influenced by any change in reaction conditions.

The synthesis of (*R*)-1-phenylethanol in the biphasic reaction systems buffer/MTBE and buffer/IL showed low conversion. With 12% it was three times higher in the MTBE system than in the IL system. For both systems *ee* was >99%.

In the continuous synthesis of (*R*)-1-phenylethanol in the system buffer/MTBE the LB-ADH proved high stability and allowed for higher conversion than in the batch system. The longer the residence time was the higher was the conversion. The same high stability was observed for the continuous synthesis of (*R*)-2-butanol. Very high TTN of >8000 for the oxidized cofactor $NADP^+$ were obtained. Depending on the flow rate conversion of up to 70% and a maximum *ee* >95% was determined. Compared to the batch experiments with strongly decreasing *ee* at racemisation rates of up to 0.2% h^{-1} and conversion of 50% at most, selectivity and conversion were higher in the continuous reaction system which proofs to be promising for further development.

6 Conclusion and Outlook

6.1 Aim

The aim of this work was the development of a continuous reaction set-up to enantioselectively synthesize short-chain alcohols using biocatalysis. This was pursued in close collaboration with the industry partner. The enantiopure short-chain alcohols were synthesized by enzymatic reduction of prochiral ketones using ADH. Especially for the short-chain substrates enzyme catalysis offers the possibility to obtain high enantioselectivity. In agreement with the industry partner a lyophilized and a liquid, specially purified preparation of the LB-ADH and a lyophilized preparation of the SADH-X2 were chosen. For enzyme dependent cofactor regeneration a NAD- and a NADP-dependent MDH were selected. First, the enzymes were kinetically characterized to screen for optimum reaction conditions and to investigate the influence of reaction conditions on activity. In a second step, the results were transferred to batch one-phase systems to proof the feasibility of synthesis of chiral alcohols, especially of (*R*)- and (*S*)-2-butanol. To overcome limitations like low conversion and decrease in *ee* the reactions were done continuously using an enzyme membrane reactor. In aqueous reaction mediums substrate range is limited by water solubility. Therefore, the reaction conditions were transferred to two-phase batch systems using MTBE and [PMIM][PF_6] as non-reactive phases. The final step was the establishment of a continuous two-phase reaction set-up for synthesis of enantiopure alcohols on larger scale based on the results obtained from the batch experiments. This way, conversion and *ee* could be positively influenced by continuous product extraction.

6.2 Kinetic Characterization

The kinetic characterization of the three enzyme preparations showed a broad substrate spectrum with good activity towards all 12 tested linear and aromatic prochiral ketones (Scheme 6-1). Activity varied greatly with the substrate ketones and was dependent on both the enzyme itself as well as on its preparation. The SADH-X2 showed an increase in activity with increasing chain length of the linear ketones while for both LB-ADH preparations no trend became apparent. Both LB-ADH preparations generally accepted bulky, i.e. aromatic and bulky linear ketones, up to a factor of 4 better than short-chain ketones. Both, the solid and the liquid LB-ADH had highest activity towards *p*-chloro acetophenone. The incongruity between the three different enzyme preparations was able to be further monitored when

investigating the dependence of activity on reaction conditions and as a function of substrate concentration. The reaction conditions varied were:

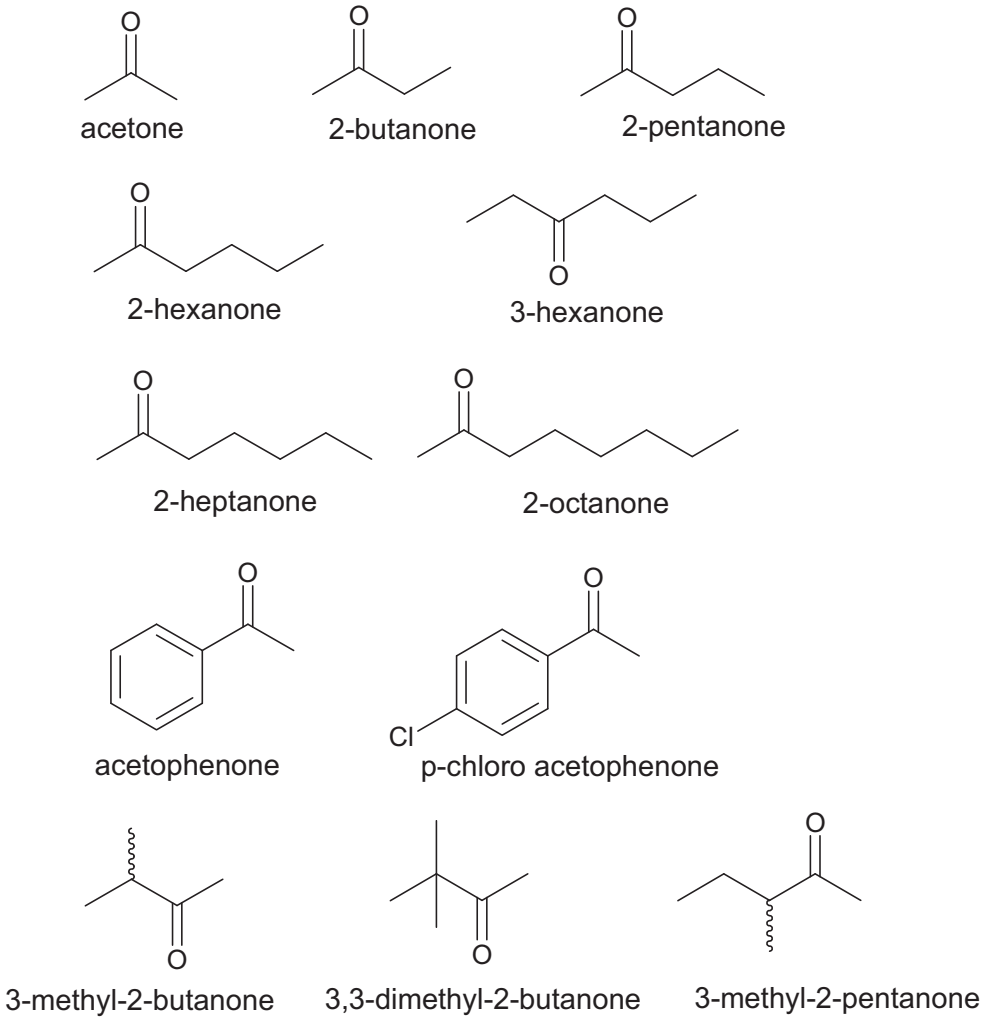
- cofactor concentration
- buffer concentration
- addition of $MgCl_2$ (ADH) and $MnCl_2$ (MDH)
- saturation of buffer with MTBE
- pH (MDH)
- *L*-malic acid concentration (MDH)

Via non-linear regression using the Michaelis-Menten equation the kinetic parameters K_M and v_{max} were determined. Where substrate excess inhibition occured additionally the inhibition constant K_S was determined by appropriate regression. The two different ADH and the two different LB-ADH preparations exhibited pronouncedly different kinetic parameters depending on the substrate and on reaction conditions (Table 6-18). E.g. the SADH-X2 showed highest v_{max} of 10.0 U mg^{-1} towards 2-butanone in presence of $MgCl_2$ whereas the solid LB-ADH was most active towards acetophenone (v_{max} = 9.7 U mg^{-1}) at the same reaction conditions. Compared to that, the liquid LB-ADH had its maximum activity of 890 U mL^{-1} in presence of acetone. Both LB-ADH preparations were least active towards 2-propanol whereas the reaction conditions were different. Similar comparisons could be made for K_M without the possibility to allow for generalization. E.g. for the SADH-X2 the saturation of the reaction medium with MTBE led to a 2-fold increase in activity in presence of acetone, but a decrease by a factor of 2.2 was observed in presence of 2-butanone. Both the solid and the liquid LB-ADH reacted with a decrease in v_{max} in presence of both substrates. The decrease was more significant for the liquid than for the solid preparation. The results indicate a strong dependence of kinetics from reaction conditions for the synthesis of enantiopure alcohols. The obtained values for K_M show that for nearly all combinations of ketone and 2-propanol, which were used as co-substrate for cofactor regeneration, an excess of 2-propanol will be needed. In view of the constant improvements of the enzyme and changes in preparation methods a full characterization of the kinetics is not advisable. Evidence for preparative reaction conditions is available from less extensive, selective kinetic measurements. The results clearly show that a kinetic characterization has to be done for every enzyme and for every specific application of the enzyme. It is impossible to draw conclusions from one ADH to another or from one preparation of the same enzyme to another preparation. A demonstration of the preparative scale applicability is pursued to allow assessment of the usefulness of the approach.

Table 6-18 Comparison of kinetic parameters

ADH	substrate	c (buffer) / mmol L^{-1}	c ($MgCl_2$) / mmol L^{-1}	MTBE	**v_{max} / U mg^{-1}**	K_M / mmol L^{-1}
SADH-X2	2-butanone	50.0	1.0	-	**10.0**	380.4
	rac-1-phenylethanol	50.0	-	saturated	**0.1**	4.8
LB-ADH (s)	acetophenone	50.0	1.0	-	**9.7**	0.7
	2-propanol	500.0	-	-	**0.5**	0.5
	2-propanol	50.0	-	saturated	**0.5**	4.4
LB-ADH (l)	acetone	50.0	-	-	**890.6 U mL^{-1}**	-
	2-propanol	50.0	-	-	**31.6 U mL^{-1}**	1.5

Besides the three ADH preparations the NAD- and the NADP-dependent MDH were kinetically characterized as they were used for enzyme dependent cofactor regeneration. The typical dependence of activity on concentrations of *L*-malic acid and cofactor was observed for both MDH with differences between them. The most important result was the highly positive influence of $MnCl_2$ on activity. An increase in activity by a factor of 42 and 29, respectively, was found. For both MDH incubation of the cofactor with $MnCl_2$ gave the best results hinting towards complex formation. This is in line with results published about the formation of cofactor complexes with metal cations. [112-114]



Scheme 6-1 Prochiral ketone substrates

6.3 One-Phase Systems

The results obtained from the kinetic characterization were transferred to batch experiments in one-phase systems to test the applicability of the SADH-X2 and the solid LB-ADH for synthesis of enantiopure (*S*)- and (*R*)-2-butanol and to investigate suitable reaction conditions for two-phase systems. The results showed a racemisation of the product alcohol and also in some cases a decrease in conversion. Both could be influenced by changes in concentration of 2-butanone, 2-propanol, and the type of cofactor regeneration. At a 100-fold excess of 2-propanol racemisation rate was higher than at a 10-fold excess while initial reaction rate and maximum reached conversion were generally higher at high 2-propanol excess. An increase in 2-butanone concentration led to higher initial reaction rates and lower racemisation rates. A change from substrate to enzyme dependent cofactor regeneration enabled for maximum conversion of up to 75% whereas racemisation was not able to be

limited. The conducted experiments clearly showed the strong dependence of conversion and selectivity on reaction conditions. Cofactor regeneration played an important role for enantioselectivity and conversion.

Especially, racemisation of the product alcohol was challenging since it already occured at low conversions. Therefore, the mechanism of racemisation was investigated employing both the SADH-X2 and the LB-ADH. Oxidation by air could be excluded since after exposure to air neither (*R*)- nor (*S*)-2-butanol showed any loss in *ee*. For the SADH-X2 conversion of (*S*)- and *rac*-2-butanol to 2-butanone of up to 30% was found even if the redox equivalent NAD^+ was missing in the reaction mixture. This hinted towards a redox equivalent or a second enzyme contained in the enzyme preparation. A change in *ee* was not observed. Similar results were found for the oxidation of (*R*)- and *rac*-2-butanol using the solid LB-ADH. Conversion was quantitative or nearly quantitative both in presence and in absence of $NADP^+$. The enantiomeric excess was shifted to (*S*) in presence of the cofactor while it was less affected in absence of $NADP^+$. Heating the enzyme solution to 45 °C before starting the reaction obviously destroyed the redox equivalent or a second enzyme since no change in *ee* was observed but conversion to the ketone was quantitative. When the experiments were carried out with the specially purified liquid LB-ADH conversion was lower and decrease in *ee* was less distinct in presence of $NADP^+$. In absence of the oxidized cofactor no conversion and no change in *ee* was observed which is in line with the assumption that a second enzyme or a redox equivalent is present in the solid non-purified preparation.

The one-phase batch reactions demonstrated the feasibility of enantioselective reduction of 2-butanone. Furthermore, the continuous reactions in the EMR showed a reasonable stability under process conditions. However, racemisation and low activity render this approach unattractive for preparative scale.

6.4 Two-Phase Systems

To overcome limitations like substrate solubility and product racemisation, and to shift equilibrium to product side by extraction of the product the conclusions made from one-phase systems regarding reaction conditions were transferred to two-phase systems. MTBE and the tailor-made IL [PMIM][PF_6] were used as non-reactive phase.

To assess the boundaries of the systems the partition of substrates and products between the aqueous reactive phase and the non-reactive phase partition coefficients P_X were determined. For all tested ketones and alcohols P_X was dependent on the concentration for both the MTBE and the IL system. The presence of 2-propanol had a large effect on P_X showing that the partitioning is a complex issue for reaction systems.

Using the solid LB-ADH in the system buffer/MTBE with substrate dependent cofactor regeneration minimized racemisation of the product alcohol to <0.01% min^{-1} and allowed for conversion of up to 65%. Generally, initial reaction rate increased with increasing 2-butanone and 2-propanol concentration. Conversion decreased with increasing substrate concentration but increased with increased 2-propanol concentration.

Employment of the liquid LB-ADH preparation showed a dependence of conversion, initial reaction rate, and racemisation rate on the ADH concentration. Compared to the solid preparation, conversion and initial reaction rate were lower, but racemisation was generally lower as well which favors the liquid over the solid preparation.

Moreover, the solid LB-ADH was applied in a reaction system with enzyme dependent cofactor regeneration using the NADP-dependent MDH. Racemisation rate, initial reaction rate, initial *ee*, and final conversion and final *ee* were all dependent on MDH and 2-butanone concentration. At constant 2-butanone concentration, i.e. variable MDH concentration, the following observations were made:

- initial reaction rate showed a maximum at 8 mg mL^{-1} MDH concentration
- racemisation rate increased linearly with increasing MDH concentration
- initial *ee* increased with increasing MDH concentration, ran towards a limiting value
- final conversion had a maximum at 8 mg mL^{-1} MDH concentration
- final *ee* had a maximum at 8 mg mL^{-1} MDH concentration

At constant MDH concentration, i.e. at variable 2-butanone concentration, the following was observed:

- initial reaction rate had a maximum at 50 mmol L^{-1} 2-butanone concentration
- racemisation rate decreased with increasing 2-butanone concentration

Racemisation rates were generally higher and conversion was generally lower than with substrate dependent cofactor regeneration. The results showed that it is possible to perform synthesis of 2-butanol using enzyme dependent regeneration but the complexity of the system increases substantially.

As model substrate acetophenone was employed to synthesize (*R*)-1-phenylethanol using the solid LB-ADH and substrate dependent cofactor regeneration. Both the reaction in the MTBE and in the IL system resulted in *ee* >99%. Conversion reached 12% and 4%, respectively, within 4 h. Again, the MTBE system showed to be more suitable for the reaction than the IL system, but conversion was much lower than for the reduction of 2-butanone.

As a substrate of commercial interest 2-hexanone was reduced to (*R*)-2-hexanol using the solid LB-ADH with *ee* of 99%. The reaction was investigated depending on the 2-propanol concentration which was used from substoichiometrical to stoichiometrical to excess amounts. Especially with substoichiometrical amounts the dependence of initial reaction rate on the co-substrate concentration was very strong. A dependence on 2-hexanone concentration was observed as well showing a maximum at 50 mmol L^{-1} which was in line with the results obtained for 2-butanone reduction. The role of the 2-propanol concentration was of high importance for the position of experimental equilibrium conversion and was very well displayed by the conducted experiments.

In a specially developed and well defined two-phase reactor the continuous production of short-chain chiral alcohols was carried out. The reduction of 2-butanone in the system buffer/MTBE using the solid LB-ADH and substrate dependent cofactor regeneration conversion of up to 70% and *ee* of up to 97% was reached. The longer the residence time was the higher conversion was obtained at decreased *ee* in accordance to the postulated mechanism of racemisation. However, racemisation was slower than in the one-phase and two-phase batch reactions. Very high stability of the LB-ADH and the cofactor were found with a productivity of 1.54 mmol mg^{-1} for the ADH and a TTN of 15300 for $NADP^+$. Similar promising results regarding enzyme and cofactor stability were found for the reduction of acetophenone. In contrast to 2-butanone, conversion hardly reached 20% while *ee* was constantly >99%. The results show that the continuous reaction set-up and the two-phase system buffer/MTBE have a high potential for the synthesis of short-chain enantiopure alcohols.

6.5 Outlook

The results indicate that for screening purposes a minimum set of reaction conditions as variable parameters, the strategy of enzyme purification, and the optimization of cofactor regeneration is of key importance. Furthermore, to fully characterize the reaction systems, *in situ* cofactor monitoring is necessary. Thereby, the mechanism of cofactor regeneration will be understood, and the optimization of this reaction step will be possible. This will be essential to exploit the potential of the reaction system.

The structure of the employed enzymes needs to be determined to allow rational approach for the optimization of enzyme activity. Especially the interaction of the cofactor with metal ions needs to be better understood in view of changes in activity.

The interaction of the LB-ADH with the phase boundary in the two-phase system buffer/MTBE is of high interest as well. For further optimization of conversion and selectivity also the respective ADH needs to be optimized by molecular biology.

In order to provide an economical process special attention has to be paid to the isolation of the product alcohol from the non-reactive phase. In the case of 2-butanol isolation, the distillation of the non-reactive phase would cause high energy costs because the product alcohol is the component with the highest boiling point. Therefore, either other solvents for the non-reactive phase need to be tested or methods of selective extraction have to be developed. In view of preparative use a scale-up or numbering-up will be necessary to produce larger amounts of the respective enantiopure alcohol.

7 Methods and Materials

7.1 Chemicals

(*R*)-2-butanol	Fluka
(*S*)-2-butanol	Fluka
[PMIM][PF_6]	TU Eindhoven
1-butanol	Fluka
2-butanone	Riedel-de Haën
2-heptanone	Aldrich
2-hexanone	Aldrich
2-octanone	Aldrich
2-pentanone	Fluka
3,3-dimethyl-2-butanone	Fluka
3-hexanone	Merck
3-methyl-2-butanone	Merck
3-methyl-2-pentanone	Aldrich
acetophenone	Fluka
H_3PO_4	Fluka
K_2HPO_4	KMF
KH_2PO_4	Riedel-de Haën
LB-ADH, liquid preparation	X-Zyme
LB-ADH, lyophilisate	X-Zyme
L-malic acid	Carl Roth
MDH, NAD-dependent	X-Zyme
MDH, NADP-dependent	X-Zyme
$MgCl_2$	Fluka
$MnCl_2$	Roth
MTBE	Merck
NAD^+	Carl Roth
NADH	Carl Roth
$NADP^+$	Carl Roth
NADPH	Carl Roth
p-chloro acetophenone	Aldrich
rac-1-phenylethanol	Fluka
rac-2-butanol	Fluka
rac-2-heptanol	Aldrich
rac-2-hexanol	Fluka
rac-2-octanol	Aldrich
rac-2-pentanol	Fluka
SADH-X2	X-Zyme
standard buffer solution, pH 4.01	Carl Roth
standard buffer solution, pH 7.01	Carl Roth

7.2 Apparatus

Cryostate	Julabo F25-ME
	Temperature: -28…200 °C
Dosage Pump	Microliter Dosage Pump MDSP3f
	Micro Mechatronic Technologies GmbH
GC	Agilent Technologies HP 6890, JAS-Unis Inlet
	CP-Chirasil-DEX CB (25 m x 0.25 mm inner diameter, film thickness 0.25 μm), Varian
	Lipodex G (25 m x 0.25 mm inner diameter, film thickness 0.25 μm), Macherey-Nagel
	Carrier gas: H_2
	Inlet temperature: 300 °C
	Detector Temperature: 280 °C
Multiplate Reader	PowerWave Spectral Photometer, BioTek Instruments
	Wavelength, 200-999 nm
	Light source: xenon flashlight
pH Meter	Model pH211, VWR Collection
Thermostate	Haake, stock of the ITMC, RWTH Aachen
Membrane Dosage Pump	Telab BF 414/32S

7.3 Gas Chromatography

Methods for gas chromatography are developed for all used substrates and the chiral product alcohols (Table 7-1). Quantitative and chiral analysis can be done using the same method. For the C_2- to C_6-alcohols the column CP-Chirasil-DEX CB, for alcohols $>C_6$ the column Lipodex G is used. The methods are developed by systematic variation of initial temperature and temperature program. For the quantitative analysis correction factors are determined giving the relation between substance area and standard area (Table 7-2). The correction factors are determined by GC analysis of defined mixtures of standard and substance using Equation 7-1. Solving the equation for $m_{substance}$ allows for the calculation of the amount of substance contained in the sample with known $m_{standard}$ and correction factor.

$$CF = \frac{m_{\text{substance}} A_{\text{standard}}}{m_{\text{standard}} A_{\text{substance}}}$$

Equation 7-1 Calculation of correction factors

Table 7-1 Methods for GC analysis

substance	t / min	T_{oven} / °C	mode	column
2-butanone	2.7	40 (3 min) 1 min^{-1} 45 (5 min) 10 min^{-1} 60 (1 min)	0.5 bar (constant)	Chirasil-DEX
2-butanol	(*R*): 6.6 (*S*): 6.9	40 (3 min) 1 min^{-1} 45 (5 min) 10 min^{-1} 60 (1 min)	0.5 bar (constant)	Chirasil-DEX
2-butanone (flow cell)	2.7	40 (3 min) 1 min^{-1} 45 (5 min)	0.5 bar (constant)	Chirasil-DEX
2-butanol (flow cell)	(*R*): 6.6 (*S*): 6.9	40 (3 min) 1 min^{-1} 45 (5 min)	0.5 bar (constant)	Chirasil-DEX
2-pentanone	4.2	50 (5 min) 1 min^{-1} 55 (5 min)	0.5 bar (constant)	Chirasil-DEX
2-pentanol	(*R*): 10.7 (*S*): 11.2	50 (5 min) 1 min^{-1} 55 (5 min)	0.5 bar (constant)	Chirasil-DEX
2-hexanone	9.4	50 (10 min)	0.5 bar (constant)	Chirasil-DEX
2-hexanol	(*R*): 14.8 (*S*): 15.0	50 (10 min)	0.5 bar (constant)	Chirasil-DEX
2-heptanone	6.8	65 (3 min) 1 min^{-1} 70 (5 min) 10 min^{-1} 80 (1 min)	0.5 bar (constant)	Chirasil-DEX
2-heptanol	12.5	65 (3 min) 1 min^{-1} 70 (5 min) 10 min^{-1} 80 (1 min)	0.5 bar (constant)	Chirasil-DEX
2-octanone	11.4	80 (3 min) 1 min^{-1} 100 (5 min)	0.5 bar (constant)	Chirasil-DEX
2-octanol	19.3	80 (3 min) 1 min^{-1} 100 (5 min)	0.5 bar (constant)	Chirasil-DEX
2-octanol	(*R*): 8.4 (*S*): 8.9	60 (4 min) 2 min^{-1} 100 (5 min)	0.5 bar (constant)	Lipodex G
Acetophenone	4.7	110 (5 min) 3 min^{-1} 125 (1 min)	1.5 mL min^{-1} (constant)	Chirasil-DEX
1-phenylethanol	(*R*): 8.9 (*S*): 9.5	110 (5 min) 3 min^{-1} 125 (1 min)	1.5 mL min^{-1} (constant)	Chirasil-DEX
acetophenone (flow cell)	4.7	110 (5 min) 3 min^{-1} 125 (1 min)	0.5 bar (constant)	Chirasil-DEX
1-phenylethanol (flow cell)	(*R*): 8.9 (*S*): 9.5	110 (5 min) 3 min^{-1} 125 (1 min)	0.5 bar (constant)	Chirasil-DEX

Table 7-2 Standards for GC analysis and correction factors

substance	standard	correction factor
2-butanone	1-butanol (11.8 min)	1.22
2-butanol	1-butanol (11.8 min)	1.08
2-pentanone	2-pentanol	1.17
2-pentanol	2-pentanone	1.13
2-hexanone	2-hexanol	1.07
2-hexanol	2-hexanone	0.93
2-heptanone	2-heptanol	0.91
2-heptanol	2-heptanone	1.11
2-octanone	2-octanol	1.02
2-octanol	2-octanone	0.98
acetophenone	*p*-chloro acetophenone (12.4 min)	0.83
1-phenylethanol	*p*-chloro acetophenone (12.4 min)	0.83

7.4 Preparation of Stock Solutions

7.4.1 Buffer Solution

For 500 mL of a 500 mmol L^{-1} potassium phosphate buffer 29.65 g (0.17 mol) KH_2PO_4 and 10.85 g (0.08 mol) K_2HPO_4 are dissolved in 300 mL de-ionized water. The pH value is adjusted to 6.5 with 85% H_3PO_4, and the volume is filled up to 500 mL with de-ionized water. For lower buffer concentrations the stock solution is diluted with de-ionized water according to the required concentration.

7.4.2 Substrate Solutions

Standard solutions, correction factors, and partition coefficients:

According to Table 7-3 the stock solutions for the standard solutions and for the determination of correction factors and partition coefficients are prepared. For the standard solutions and for the correction factors acetone is used as solvent. For the partition coefficients the stock solutions are prepared using the solvent for the non-reactive phase, i.e. MTBE or [PMIM][PF_6]. The respective volume of the substance is added to the solvent, weight, and the actual concentration is calculated. For the partition coefficients the non-aqueous phases are saturated with water. The stock solutions are diluted with the respective solvent to the needed concentrations.

Table 7-3 Preparation of stock solutions for standard solutions and determination of correction factors and partition coefficients

substance	V / mL	c / mmol L^{-1}	n / mmol	V (substance) / μL	m (substance) / mg
2-butanone	5.0	500.0	2.5	223.9	180.3
2-butanol	5.0	500.0	2.5	228.8	185.3
1-butanol	5.0	500.0	2.5	228.8	185.3
2-pentanone	5.0	500.0	2.5	265.8	215.3
2-pentanol	5.0	500.0	2.5	272.1	220.4
2-hexanone	5.0	500.0	2.5	308.4	250.4
2-hexanol	5.0	500.0	2.5	315.3	255.4
2-heptanone	5.0	500.0	2.5	348.1	285.5
2-heptanol	5.0	500.0	2.5	355.6	290.5
2-octanone	5.0	500.0	2.5	391.9	320.6
2-octanol	5.0	500.0	2.5	397.5	325.6
acetophenone	5.0	500.0	2.5	291.6	300.4
1-phenylethanol	5.0	500.0	2.5	302.4	305.4
p-chloro acetophenone	5.0	500.0	2.5	324.8	386.5

Solutions for kinetic measurements and for batch and continuous experiments:

According to Table 7-4 the stock solutions for the kinetic measurements and for the batch and continuous experiments are prepared. For the kinetic measurements and for the experiments in one-phase systems the potassium phosphate buffer is used as solvent. To prepare the stock solutions needed for experiments in a two-phase system the respective substrate is dissolved in the non-aqueous phase, i.e. MTBE or [PMIM][PF_6]. The solvents for the two-phase reactions are saturated with one another.

Table 7-4 Preparation of stock solutions for kinetic measurements, batch and continuous experiments

substance	V / mL	c / mmol L^{-1}	n / mmol	V (substance) / µL	m (substance) / mg
acetone	100.0	1000.0	100.0	7351.9	5808.0
2-propanol	100.0	1000.0	100.0	7825.5	6010.0
2-butanone	100.0	500.0	50.0	4478.9	3605.5
2-butanol	100.0	500.0	50.0	4575.3	3706.0
2-pentanone	100.0	40.0	4.0	425.3	344.5
2-hexanone	100.0	40.0	4.0	493.4	400.6
3-hexanone	100.0	40.0	4.0	494.6	400.6
acetophenone	100.0	20.0	2.0	233.3	240.3
1-phenylethanol	100.0	100.0	10.0	1209.6	1221.7
p-chloro acetophenone	100.0	20.0	2.0	259.8	309.2
3-methyl-2-butanone	100.0	40.0	4.0	430.7	344.5
3,3-dimethyl-2-butanone	100.0	40.0	4.0	498.9	400.6
3-methyl-2-pentanone	100.0	40.0	4.0	491.6	400.6

7.5 Kinetic Measurements

The SADH-X2, the solid and the liquid LB-ADH preparation, the NAD-dependent and the NADP-dependent MDH are kinetically characterized by variation of reaction parameters like concentration of cofactor, substrate, buffer, and by addition of $MgCl_2$ and $MnCl_2$. The reactions cause a decrease or increase in cofactor concentration which can be monitored by UV/Vis measurements at 340 nm. Using Lambert-Beer's law (Equation 7-2) the enzyme activity can be calculated from the measurements (Equation 7-3).

$$A = c \cdot d \cdot \varepsilon$$

Equation 7-2 Lambert-Beer's law

$$v = \frac{dc}{dt} = \frac{1}{\varepsilon \cdot d} \cdot \frac{dA}{dt} = \frac{m}{\varepsilon \cdot d}$$

A = absorption
c = concentration
d = layer thickness
ε = extinction coefficient
v = reaction rate
t = time
m = slope

Equation 7-3 Calculation of reaction rate v using Lambert-Beer's law

All measurements are conducted using 96-well plates with the multiplate reader supplied by BioTek Instruments. The parameters for the measurements are as follows:

5 seconds shaking
measurement: 2.3 min
interval: 2 seconds
T = 30 °C
V = 220 μL

7.5.1 Alcohol Dehydrogenases

The standard assay is defined as follows:

c (cofactor) = 0.5 mmol L^{-1}
c (buffer) = 50 mmol L^{-1}
c (SADH-X2) = 0.04 mg mL^{-1}
c ($LB\text{-}ADH_{solid}$) = 0.04 mg mL^{-1}
c ($LB\text{-}ADH_{liquid}$) = 0.9 μL mL^{-1}
pH = 6.5

For concentration of substrate stock solutions see Table 7-4. Other stock solutions:

cofactor: 10.0 mg mL^{-1}
SADH-X2 solution: 0.08 mg mL^{-1}
LB-ADH_{solid} solution: 0.08 mg mL^{-1}
LB-ADH_{liquid} solution: 10.0 mL mL^{-1}

For variation of the cofactor concentration the respective volume of the stock solution is added to the assay to achieve the needed concentration. The reactions are started by addition of enzyme. Well H of a row A to H always contains the blank solution which does not contain any enzyme.

x μL cofactor solution
100-x μL buffer
100 μL 50 mmol L^{-1} substrate solution
20 μL of 1:5 dilution of ADH solution

For kinetic measurements depending on the substrate concentration dilution series are made in the multiplates. In wells B to G 100 μL buffer are pipetted. To wells A and B 200 μL of the stock solution are added. From well B 100 μL are taken after mixing and added to well C and so on. Well H contains the blank solution composed of 200 μL buffer and cofactor. To wells A to G 100 μL enzyme solution are added. The reaction is always started by addition of 20 μL cofactor solution. For measurements done in presence of 1.0 mmol L^{-1} $MgCl_2$ all solutions used for the measurements contain 1.0 mmol L^{-1} $MgCl_2$.

7.5.2 Malate Dehydrogenases

The dilution series for measurements with variable *L*-malic acid and variable cofactor concentration are prepared according to the instructions for the ADH in Chapter 7.5.1. For measurements conducted in the presence of $MnCl_2$ 100 μL of a 2.0 mmol L^{-1} $MnCl_2$ solution in the respective buffer are added instead of pure buffer.

Concentrations of stock solutions:

cofactor:	5.0 mg mL^{-1}
MDH solution:	0.08 mg mL^{-1}
LB-ADH_{solid} solution:	0.08 mg mL^{-1}
LB-ADH_{liquid} solution:	10.0 mL mL^{-1}
$MnCl_2$ solution:	2.0 mmol L^{-1}

Concentrations for the measurements:

c (buffer)	= 50 mmol L^{-1}
c (MDH)	= 0.1 mg mL^{-1}

7.6 Partition Coefficients

For the preparation of the stock solutions (Table 7-3) MTBE and [PMIM][PF_6] are saturated with water. The partition coefficients are composed according to Table 7-5 to Table 7-8. The water used is saturated with the respective non-reactive phase. The reaction vessels are thoroughly closed with Parafilm, mixed for 30 seconds using a Vortex, and stored at 30 °C for four days. From each phase 50 μL samples are taken and 100 μL of the respective GC standard are added. The samples are measured three times each, and the concentrations of each phase are calculated from the GC measurements. The values for P_X are calculated using Equation 5-1.

Table 7-5 Composition of water/MTBE system for determination of P_X for ketones

ketone	c (ketone) / mmol L^{-1}	V (stock solution) / mL	V (MTBE) / mL	V (water) / mL
2-butanone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
acetophenone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-pentanone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-hexanone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-heptanone	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-octanone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0

Table 7-6 Composition of water/[PMIM][PF_6] system for determination of P_X for ketones

ketone	c (ketone) / mmol L^{-1}	V (stock solution) / mL	V ([PMIM][PF_6]) / mL	V (water) / mL
2-butanone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
acetophenone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-pentanone	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-hexanone	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-heptanone	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-octanone	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0

Table 7-7 Composition of water/MTBE system for determination of P_X for alcohols

alcohol	c (alcohol) / mmol L^{-1}	V (stock solution) / mL	V (MTBE) / mL	V (water) / mL
2-butanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
1-phenylethanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-pentanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-hexanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-heptanol	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-octanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0

Table 7-8 Composition of water/[PMIM][PF_6] system for determination of P_X for alcohols

alcohol	c (alcohol) / mmol L^{-1}	V (stock solution) / mL	V ([PMIM][PF_6]) / mL	V (water) / mL
2-butanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
1-phenylethanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-pentanol	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-hexanol	20.0	0.2	1.8	2.0
	40.0	0.4	1.6	2.0
	60.0	0.6	1.4	2.0
	80.0	1.0	1.0	2.0
	160.0	1.6	0.4	2.0
2-heptanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0
2-octanol	10.0	0.1	1.9	2.0
	20.0	0.2	1.8	2.0
	30.0	0.3	1.7	2.0
	50.0	0.5	1.5	2.0
	80.0	0.8	1.2	2.0

7.7 Experiments using One-Phase Systems

7.7.1 Batch Experiments

The reaction mixtures are composed according to the concentrations and reaction volumes given in Table 7-9. The needed amounts of enzymes and cofactors are dissolved in 0.5 mL of the respective 50 mmol L^{-1} or 100 mmol L^{-1} buffer. The reactions are always started by addition of the enzyme solution and are stirred slowly. Samples of 50 μL are taken regularly and mixed with 100 μL of the respective standard solution. Conversion and *ee* are calculated from the GC data (Equation 7-1). The reactions are conducted at room temperature.

7.7.2 Continuous Experiments

The used enzyme membrane reactor (EMR) is made of poly-ether ether ketone (PEEK). Its total volume is 10 mL. Below the membrane a magnetic stirring bar made of Teflon is located. The EMR is connected to a thermostat to maintain the reactor at 30 °C. Additionally, the stock solution is cooled to 4 °C using a cryostat. The substrate solution is pumped into the reactor using a membrane dosage pump produced by Telab. The pump is connected to the reactor with a 1/16” tube. The reactor outlet is connected to the flow cell of the GC autosampler using a 1/16” tube. The product mixture leaving the flow cell is collected.
For starting up the reactor is filled with substrate solution. A commercial ultra filtration membrane made of poly-ether sulfone is fitted to the reactor avoiding bubbles. The membrane has a rejection limit of 10 kDa. The membrane holds back the enzyme but lets pass through the substrates, products, and the cofactor. The reactor is closed and tested for leakages. Afterwards, 1.0 mL of a 20 mg mL^{-1} solution of ovalbumin in buffer is pumped into the reactor to cover the membrane and avoid enzyme adsorption on the membrane. To ensure the complete covering of the membrane substrate solution is pumped through the reactor for several minutes. The reaction is started by first pumping the MDH and then the SADH-X2 solution into the reactor. The autosampler is started and GC samples are measured over the whole reaction time.

7.8 Experiments using Two-Phase Systems

7.8.1 Batch Experiments

The reaction mixtures are composed according to the concentrations and reaction volumes given in Table 7-9. The stock solutions are prepared using the non-reactive phases as solvents. The needed amounts of enzymes and cofactors are dissolved in 0.5 mL of the respective 50 mmol L^{-1} or 100 mmol L^{-1} buffer. The reactions are always started by addition of the enzyme solution and are stirred slowly. Samples of 50 μL are regularly taken from the non-reactive phase and mixed with 100 μL of the suitable standard solution. To ensure equal phase volumes in spite of sample taking 50 μL are taken out of the aqueous phase with each sample and discarded. Conversion and *ee* are calculated from the GC data (Equation 7-1). The reactions are conducted at room temperature.

7.8.2 Continuous Experiments

The reaction vessel has a total volume of 10 mL. The reactive and the non-reactive phase are stirred independently, so the composition of each 5.0 mL phase is uniform. Using a cryostat the reactor is constantly held at 30 °C. A substrate reservoir containes the feed substrate solution which is substrate dissolved in MTBE. A thermostat is employed to maintain it at 4.0 °C. A syringe pump feeds the substrate solution into the reactive phase of the reactor while it transfers the identical volume out of the reactor through the GC flow cell and into a waste bottle. At intermittent time intervals, the GC autosampler removes 1 μL of a standard 100 mmol L^{-1} 1-butanol solution before injecting the sample and standard together into the GC for analysis.

7.9 Batch Synthesis of (*R*)-2-Butanol using Two-Phase Systems

7.9.1 Buffer/MTBE

Solid LB-ADH:

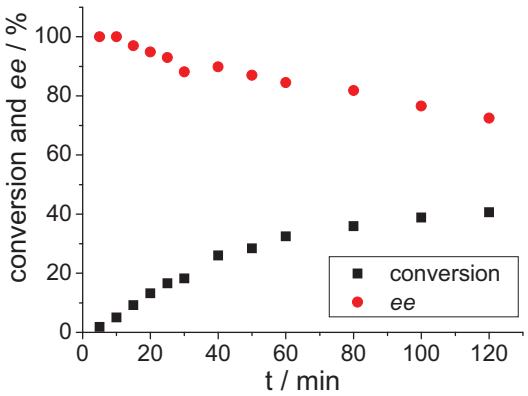


Figure 7-1 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 20 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 09)

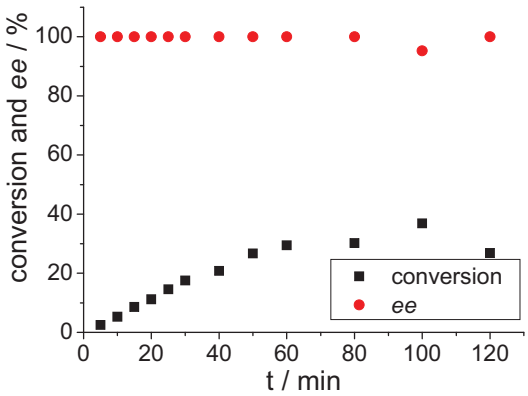


Figure 7-2 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 20 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 10)

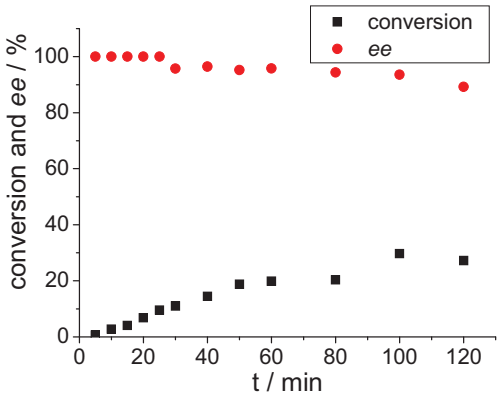


Figure 7-3 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 03)

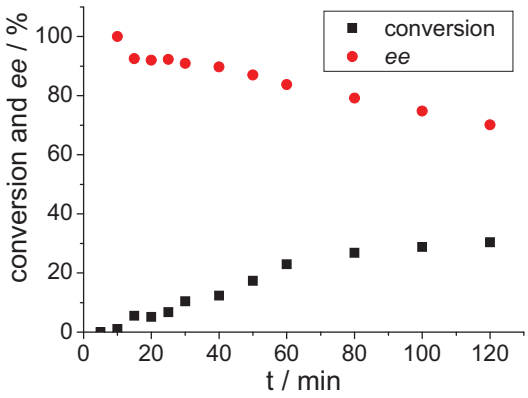


Figure 7-5 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 100 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 11)

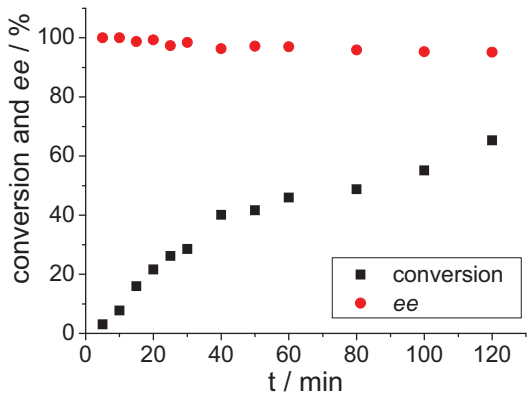


Figure 7-4 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 05)

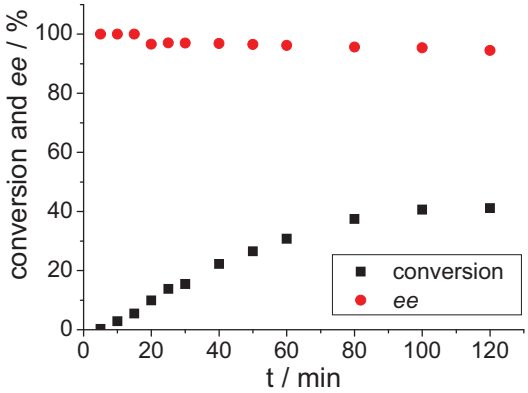


Figure 7-6 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 100 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 12)

Liquid LB-ADH:

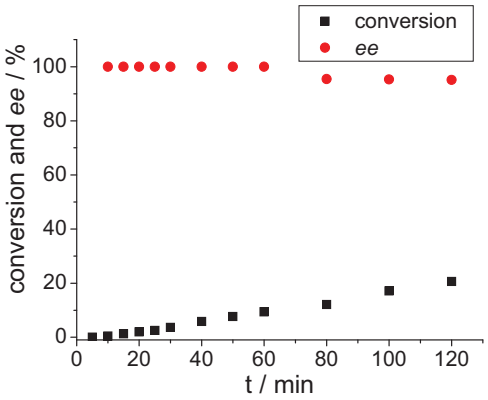


Figure 7-7 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 5.0 µL mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 18)

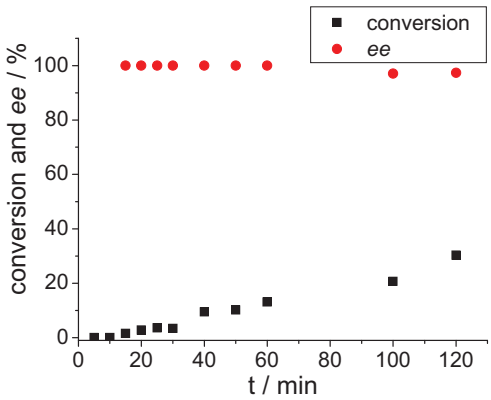


Figure 7-8 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 5.0 µL mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 23)

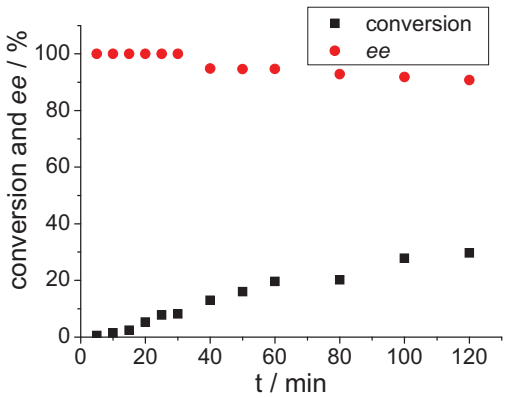


Figure 7-9 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 10.0 µL mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 19)

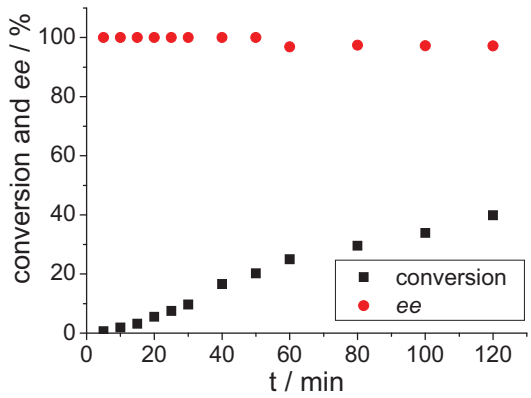


Figure 7-10 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 10.0 µL mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 24)

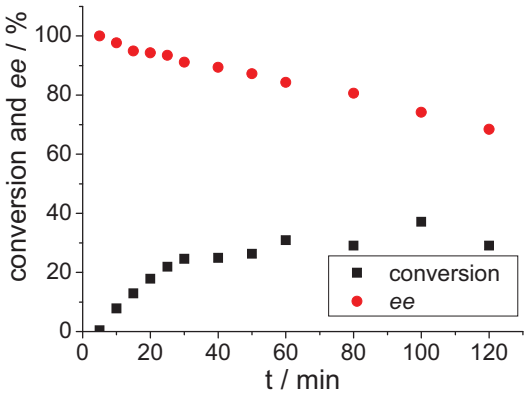


Figure 7-11 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 20.0 µL mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 04)

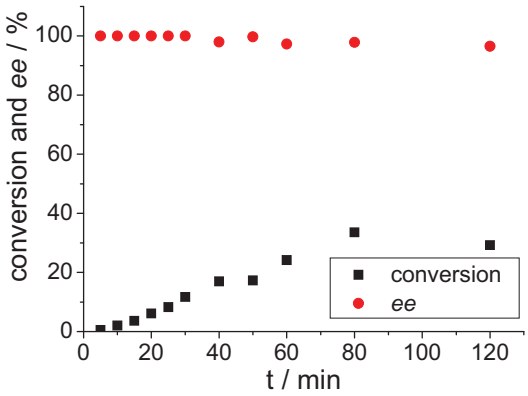


Figure 7-12 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 20.0 µL mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 06)

7.9.2 Enzyme-Dependent Cofactor Regeneration

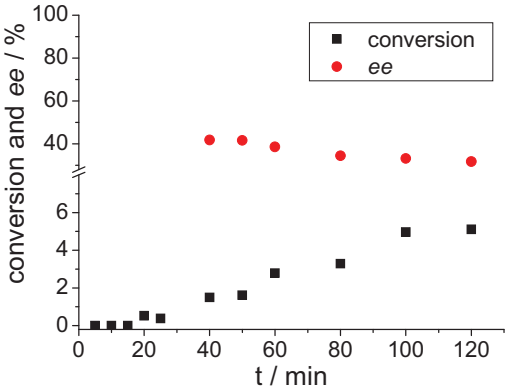


Figure 7-13 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 13)

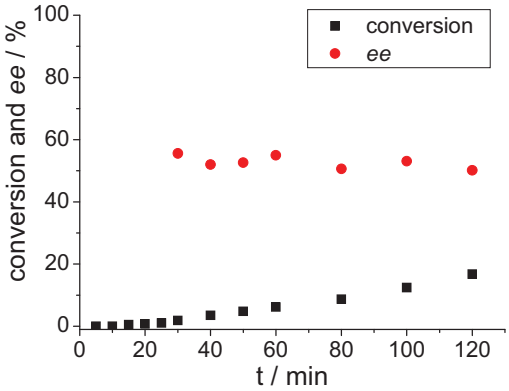


Figure 7-14 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 2.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 14)

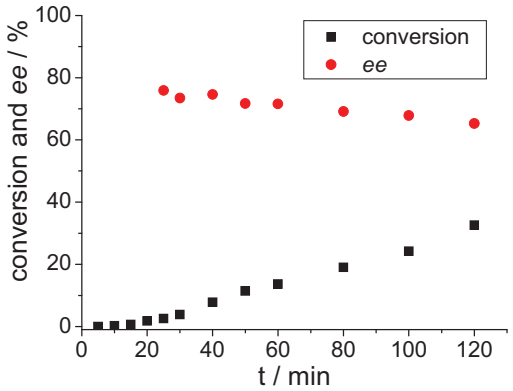


Figure 7-15 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 4.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 15)

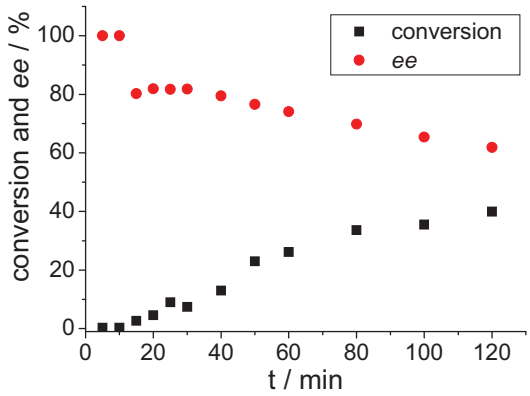


Figure 7-16 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 6.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 20)

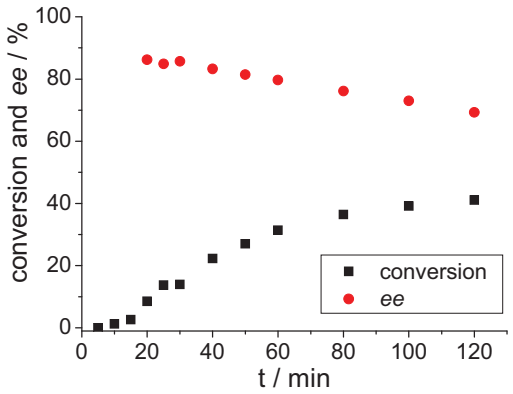


Figure 7-17 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 8.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 16)

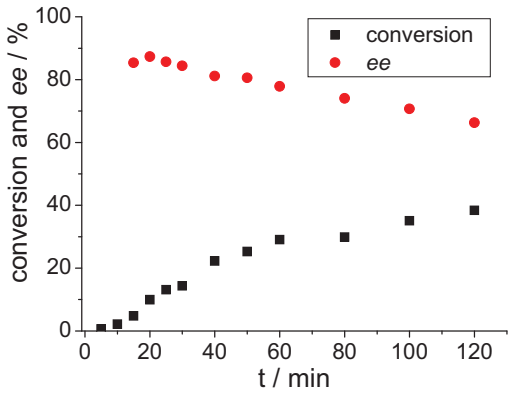


Figure 7-18 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 10.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 17)

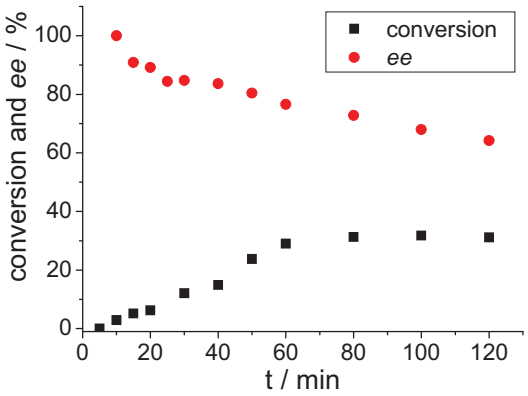


Figure 7-19 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 50 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 12.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 21)

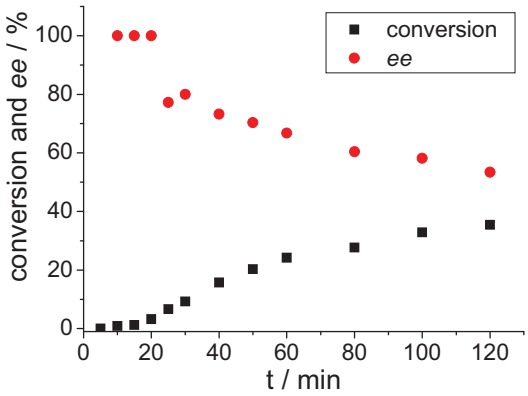


Figure 7-20 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 20 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 8.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 26)

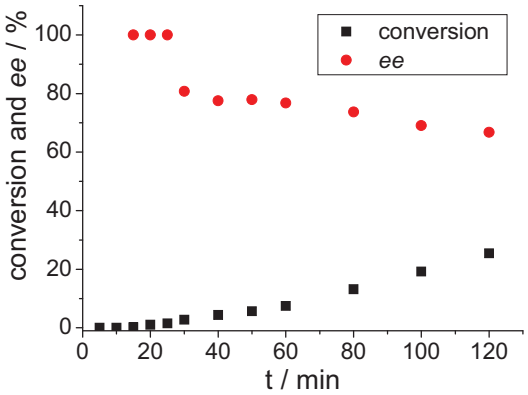


Figure 7-21 Batch synthesis of (*R*)-2-butanol in the system buffer/MTBE (c (2-butanone) = 100 mmol L^{-1}, c (*L*-malic acid) = 60 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (MDH) = 8.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: BB 25)

7.9.3 Buffer/[PMIM][PF_6]

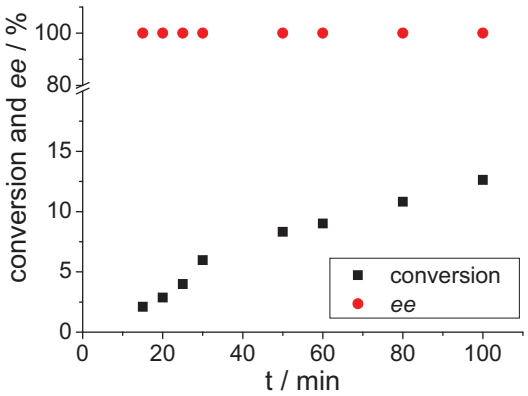


Figure 7-22 Batch synthesis of (*R*)-2-butanol in the system buffer/[PMIM][PF_6] (c (2-butanone) = 20 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: PM 167)

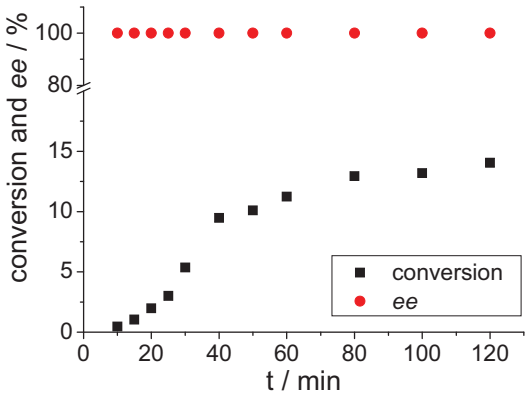


Figure 7-23 Batch synthesis of (*R*)-2-butanol in the system buffer/[PMIM][PF_6] (c (2-butanone) = 20 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: AL 20)

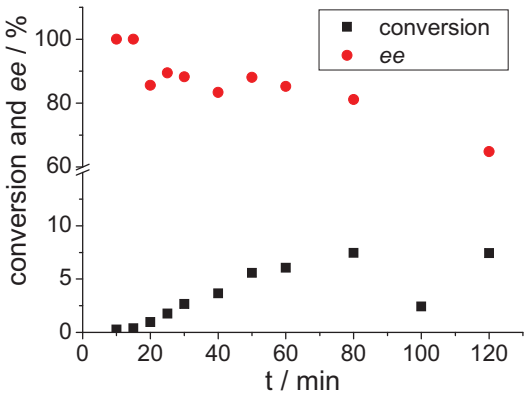


Figure 7-24 Batch synthesis of (*R*)-2-butanol in the system buffer/[PMIM][PF_6] (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: AL 12)

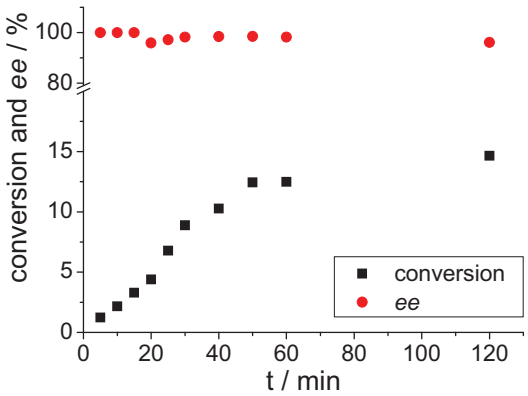


Figure 7-25 Batch synthesis of (*R*)-2-butanol in the system buffer/[PMIM][PF_6] (c (2-butanone) = 50 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: AL 10)

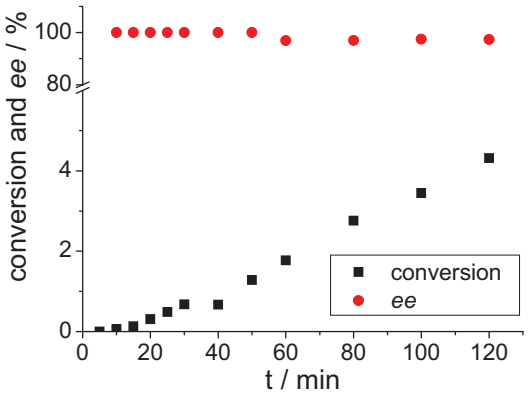


Figure 7-26 Batch synthesis of (*R*)-2-butanol in the system buffer/[PMIM][PF_6] (c (2-butanone) = 100 mmol L^{-1}, c (2-propanol) = 0.2 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: AL 19)

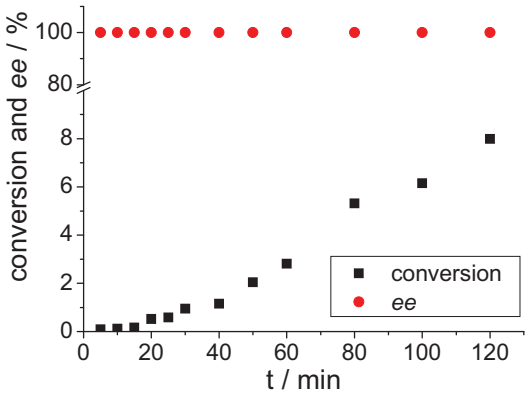


Figure 7-27 Batch synthesis of (*R*)-2-butanol in the system buffer/[PMIM][PF_6] (c (2-butanone) = 100 mmol L^{-1}, c (2-propanol) = 2.0 mol L^{-1}, c ($NADP^+$) = 0.1 mmol L^{-1}, c (LB-ADH) = 1.0 mg mL^{-1}, c (buffer) = 50 mmol L^{-1}, V (reaction) = 5.0 mL, experiment: AL 1)

Table 7-9 Overview of experiments

exp.	subst.	c (subst.) / mmol L^{-1}	c (2-propanol) / mol L^{-1}	c (*L*-malic acid) / mmol L^{-1}	c ($NADP^+$) / mmol L^{-1}	V (phase) / mL	non-reactive phase	ADH	c (ADH) / mg mL^{-1}	2nd enzyme	c (2nd enzyme) / mg mL^{-1}	c (buffer) / mmol L^{-1}
PM 154	BuO	20.0	0.2	-	0.1 (NAD^+)	1.0	-	SADH-X2	1.0		-	100.0
PM 155	BuO	20.0	2.0	-	0.1 (NAD^+)	1.0	-	SADH-X2	1.0		-	100.0
PM 167	BuO	20.0	0.2	-	0.1	5.0	[PMIM][PF_6]	LB-ADH	1.0			50.0
PM 174	BuO	20.0	0.2	-	0.1	5.0	-	LB-ADH	1.0			50.0
PM 178	BuO	20.0	2.0	-	0.1	5.0	-	LB-ADH	1.0			50.0
PM 179	BuO	100.0	0.2	-	0.1	5.0	-	LB-ADH	1.0			50.0
PM 180	BuO	100.0	2.0	-	0.1	5.0	-	LB-ADH	1.0			50.0
PM 197	(*R*)-BuOH	10.0	0.2	-	0.1	5.0	-	LB-ADH	2.5 μL mL^{-1}	-	-	50.0
PM 198	*rac*-BuOH	10.0	0.2	-	0.1	5.0	-	LB-ADH	2.5 μL mL^{-1}	-	-	50.0
PM 199	(*R*)-BuOH	10.0	0.2	-	-	5.0	-	LB-ADH	2.5 μL mL^{-1}	-	-	50.0
PM 200	*rac*-BuOH	10.0	0.2	-	-	5.0	-	LB-ADH	2.5 μL mL^{-1}	-	-	50.0
AL 8	BuO	50.0	0.2	-	0.1	5.0	-	LB-ADH	1.0			50.0
AL 9	BuO	50.0	2.0	-	0.1	5.0	-	LB-ADH	1.0			50.0
AL 10	BuO	50.0	2.0	-	0.1	5.0	[PMIM][PF_6]	LB-ADH	1.0			50.0
AL 12	BuO	50.0	0.2	-	0.1	5.0	[PMIM][PF_6]	LB-ADH	1.0			50.0
AL 18	BuO	100.0	2.0	-	0.1	5.0	[PMIM][PF_6]	LB-ADH	1.0			50.0
AL 19	BuO	100.0	0.2	-	0.1	5.0	[PMIM][PF_6]	LB-ADH	1.0			50.0
AL 20	BuO	25.0	2.0	-	0.1	5.0	[PMIM][PF_6]	LB-ADH	1.0			50.0
BB 03	BuO	50.0	0.2	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 04	BuO	50.0	0.2	-	0.1	5.0	MTBE	LB-ADH	20 μL mL^{-1}	-	-	50.0
BB 05	BuO	50.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 06	BuO	50.0	2.0	-	0.1	5.0	MTBE	LB-ADH	20 μL mL^{-1}	-	-	50.0
BB 09	BuO	20.0	0.2	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0

exp.	subst.	c (subst.) / mmol L^{-1}	c (2-propanol) / mol L^{-1}	c (*L*-malic acid) / mmol L^{-1}	c ($NADP^+$) / mmol L^{-1}	V (phase) / mL	non-reactive phase	ADH	c (ADH) / mg mL^{-1}	2nd enzyme	c (2nd enzyme) / mg mL^{-1}	c (buffer) / mmol L^{-1}
BB 10	BuO	20.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 11	BuO	100.0	0.2	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 12	BuO	100.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 13	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	1.0	50.0
BB 14	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	2.0	50.0
BB 15	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	4.0	50.0
BB 16	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	8.0	50.0
BB 17	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	10.0	50.0
BB 18	BuO	50.0	0.2	-	0.1	5.0	-	LB-ADH	5 $\mu L\ mL^{-1}$	-	-	50.0
BB 19	BuO	50.0	0.2	-	0.1	5.0	-	LB-ADH	10 $\mu L\ mL^{-1}$	-	-	50.0
BB 20	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	6.0	50.0
BB 21	BuO	50.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	12.0	50.0
BB 23	BuO	50.0	2.0	-	0.1	5.0	-	LB-ADH	5 $\mu L\ mL^{-1}$	-	-	50.0
BB 24	BuO	50.0	2.0	-	0.1	5.0	-	LB-ADH	10 $\mu L\ mL^{-1}$	-	-	50.0
BB 25	BuO	100.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	8.0	50.0
BB 26	BuO	20.0	-	60.0	0.1	5.0	MTBE	LB-ADH	1.0	MDH	8.0	50.0
BB 30	BuO	50.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 32	BuO	50.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 34	BuO	100.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 38	AP	50.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
BB 39	AP	50.0	2.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 01	2-HexO	20.0	0.0002	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 02	2-HexO	20.0	0.002	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 03	2-HexO	50.0	0.0002	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 04	2-HexO	50.0	0.002	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 05	2-HexO	100.0	0.0002	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 06	2-HexO	100.0	0.002	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 07	2-HexO	20.0	0.0002	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 08	2-HexO	20.0	0.002	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 09	2-HexO	50.0	0.0002	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0

exp.	subst.	c (subst.) / mmol L^{-1}	c (2-propanol) / mol L^{-1}	c (*L*-malic acid) / mmol L^{-1}	c ($NADP^+$) / mmol L^{-1}	V (phase) / mL	non-reactive phase	ADH	c (ADH) / mg mL^{-1}	2nd enzyme	c (2nd enzyme) / mg mL^{-1}	c (buffer) / mmol L^{-1}
DM 10	2-HexO	50.0	0.002	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 11	2-HexO	100.0	0.0002	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 12	2-HexO	100.0	0.002	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 13	2-HexO	20.0	0.02	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 14	2-HexO	50.0	0.05	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 15	2-HexO	100.0	0.1	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 16	2-HexO	20.0	0.2	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 17	2-HexO	50.0	0.5	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 18	2-HexO	100.0	1.0	-	0.1	5.0	MTBE	LB-ADH	1.0	-	-	50.0
DM 19	2-HexO	20.0	0.02	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 20	2-HexO	50.0	0.05	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 21	2-HexO	100.0	0.1	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 22	2-HexO	20.0	0.2	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 23	2-HexO	50.0	0.5	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
DM 24	2-HexO	100.0	1.0	-	0.1	5.0	MTBE	LB-ADH	0.5	-	-	50.0
SA 01	(*R*)-BuOH	10.0	0.2	-	0.1 (NAD^+)	5.0	-	SADH-X2	0.25	-	-	50.0
SA 02	*rac*-BuOH	10.0	0.2	-	0.1 (NAD^+)	5.0	-	SADH-X2	0.25	-	-	50.0
SA 03	(*R*)-BuOH	10.0	0.2	-	-	5.0	-	SADH-X2	0.25	-	-	50.0
SA 04	*rac*-BuOH	10.0	0.2	-	-	5.0	-	SADH-X2	0.25	-	-	50.0
TH 01	2-BuO	25.0	-	30.0	0.1 (NAD^+)	1.0	-	SADH-X2	1.0	MDH	2.5	100.0
TH 02	2-BuO	25.0	-	60.0	0.1 (NAD^+)	1.0	-	SADH-X2	5.0	MDH	5.0	100.0
TH 03	2-BuO	25.0	-	60.0	0.1 (NAD^+)	1.0	-	SADH-X2	8.0	MDH	8.0	100.0
TH 04	(*R*)-BuOH	10.0	0.2	-	0.1	5.0	-	LB-ADH	0.25	-	-	50.0
TH 05	*rac*-BuOH	10.0	0.2	-	0.1	5.0	-	LB-ADH	0.25	-	-	50.0
TH 06	(*R*)-BuOH	10.0	0.2	-	-	5.0	-	LB-ADH	0.25	-	-	50.0
TH 07	*rac*-BuOH	10.0	0.2	-	-	5.0	-	LB-ADH	0.25	-	-	50.0
TH 08	2-BuO	25.0	-	60.0	0.1 (NAD^+)	10.0	-	SADH-X2	2.0	MDH	2.5	100.0
TH 09	2-BuO	25.0	-	60.0	0.1 (NAD^+)	10.0	-	SADH-X2	2.0	MDH	2.5	100.0

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9 Appendix

9.1 List of Figures

9.2 List of Schemes

9.3 List of Tables

9.4 List of Equations

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