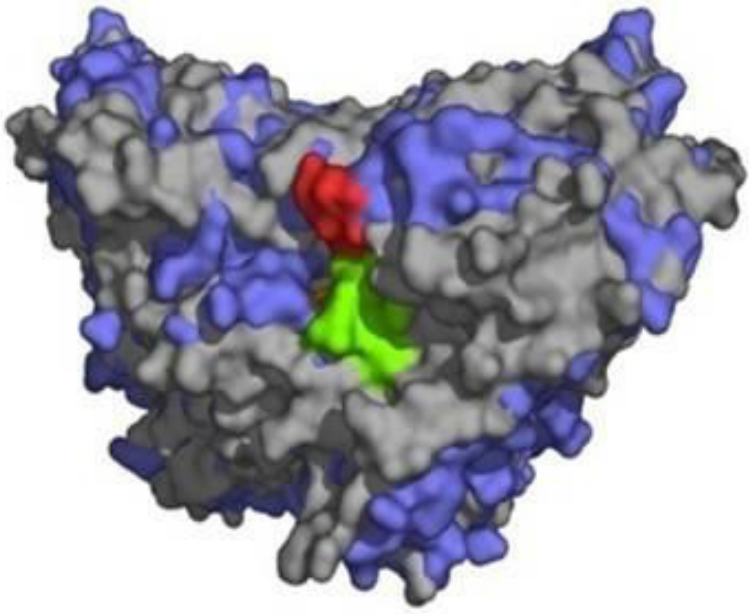
HEINRICH HEINE
UNIVERSITÄT
DÜSSELDORF

Characterization of benzaldehyde lyase and benzoylformate decarboxylase in non-conventional media



Mariya Kokova

Characterization of benzaldehyde lyase and benzoylformate decarboxylase in non-conventional media

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Mariya Kokova

aus Varna

Düsseldorf, Mai, 2009

aus dem Institut für Molekulare Enzymtechnologie

der Heinrich-Heine Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Martina Pohl

Koreferent: Prof. Dr. Jörg Pietruszka

Tag der mündlichen Prüfung: 27 Mai 2009

Contents

List of figures

List of tables

Abbreviations

ADH	alcohol dehydrogenase
APS	amonium peroxodisulphate
BAL	benzaldehyde lyase
Benzoin-ThDP	benzoin-thiamine diphosphate intermediate
BFD	benzoylformate decarboxylase
BMIM.BF_4	1-butyl-3-methylimidazolium tetrafluoroborate
BuPy	butylpyridinium
DMBA	3,5-dimethoxybenzaldehyde
DMSO	dimethyl sulfoxide
ee	enantiomeric excess
Ecoeng 1111P	1,3-dimethyl-imidazolium dimethylphosphate
Ecoeng 21M	1-ethyl-3-methyl-imidazolium iethyleneglycolmonomethylethersulfate
EHMPES	1-ethyl-3-hydroxymethylpyridinium ethylsulfate
EMIM	1-ethyl-3-methylimidazolium
EtPy	ethylpyridinium
Hbz-ThDP	hydroxybenzyl-thiamine diphosphate intermediate
HCD	high cell density
HL-ADH	horse liver alcohol dehydrogenase
HPLC	high pressure liquid chromatography
2-HPP	2-hydroxypropiophenone
IL	ionic liquid
kDa	kilodalton
LB medium	Luria-Bertani medium
IPTG	isopropyl-β-thio-galactoside
mdeg	millidegree
MTBE	methyl *tert*-butyl ether

OD	optical density
PEG	polyethylene glycol
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
ThDP	thiamine diphosphate
T_m	enzyme melting point
TMB	3,3',5,5'-tetramethoxybenzoin
U	activity unit
UV	ultra violet light
vol%	volume percent

A	concentration of first binding substrate	(mM)
k	micro-reaction constant	(s^{-1}), (mM^{-1} s^{-1})
K_{catf}	maximum turnover number	(s^{-1})
Keq	equilibrium constant	(mM^{-1})
K_{iA}	inhibition constant of the first binding substrate	(mM)
K_{iB}	inhibition constant of the second binding substrate	(mM)
K_{mA}	Michaelis constant for the first binding substrate	(mM)
K_{mB}	Michaelis constant for the second binding substrate	(mM)
K_{mP}	Michaelis constant for the product	(mM)
P	concentration of product	(mM)

I. INTRODUCTION

I.1. Why biocatalysis?

Enzymes are catalytically active proteins produced by all living organisms. The use of microorganisms as enzyme sources was already widespread among ancient people and was based mainly on empirical observations. Later on, in the eighteenth and nineteenth centuries the scientists started to study the enzymes in a more systematic way. Today enzymes are routinely used for the production of sugar syrups, cheese, beverage, animal feed, detergents, pulp, paper, textiles, various organic compounds, antibiotics, etc (**Kirk** et al., 2002; **Rodriguez Couto** and **Toca Herrera**, 2006; **Dhawan** and **Kaur**, 2007).

Compared to chemical catalysts, enzymes offer many advantages such as low energy consumption, lower waste production and biodegradability. The reason for this is the ability of enzymes to function under mild conditions in terms of temperature, pH, and solvents (**Faber**, 1997). In addition, many enzymes exhibit unique stability towards extreme temperature, pH, organic solvents and salt content which further extends their potential as catalysts (**Bruins** et al., 2001; **Demirjian** et al., 2001).

Enzymes are very efficient catalysts as they typically accelerate reactions by a factor of 10^8-10^{10}. Moreover, many enzymes are applied preferably for the production of chiral compounds as they show nearly 100 % regio- and enantioselectivity (**Faber**, 1997).

I.2. Biocatalysis in non-conventional media

Historically, the enzymatic catalysis has been carried out mainly in aqueous systems which have been considered as one of the requirements and the only possible media for enzymatic reactions (**Krishna**, 2002); therefore, the aqueous systems are traditionally regarded as ***conventional*** (or natural) reaction media. In contrast, organic solvents, ionic liquids, supercritical fluids and gases, as well as mixtures of those substances with water are regarded as ***non-conventional***.

Intriguingly, biotransformations using enzymes in non-aqueous media did not only show that enzymes can work in presence of organic solvents or even in almost water-free environment, but in some cases the enzymatic performance was even better compared to that in aqueous media. Examples include enhanced thermostability of lysosyme in glycerol, improved enantioselectivity of subtilisin in presence of DMSO, accelerated activity of horse radish peroxidase in presence of 30 vol% organic solvents, etc (**Castro** and **Knubovets**, 2003).

One feature all non-conventional media have in common is the reduced water content compared to aqueous media. According to the water content non-conventional media can be categorized as follows:

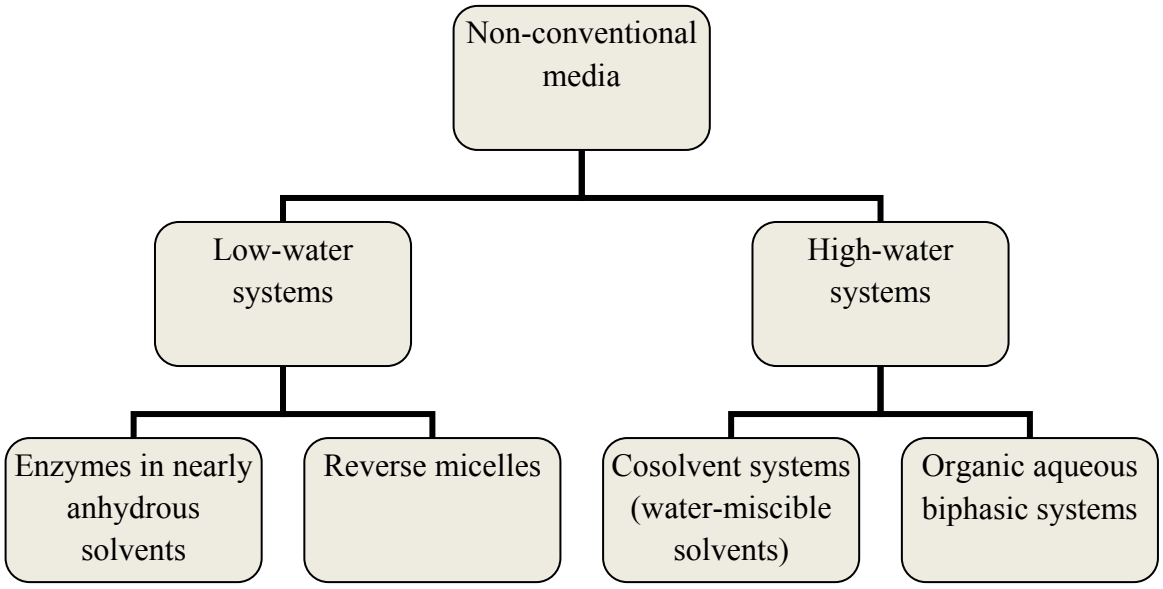


Figure 1: Classification of non-conventional media according to the water content (Gupta, 1992).

Although **Gupta** (1992) presents only four main types of systems according to the water content, the variety of solvents that can be applied is huge. It includes organic solvents and ionic liquids both water-miscible and water-immiscible, supercritical fluids and gases. Taking into account the possibility to apply mixtures of two or more of these reaction media as mono- or biphasic systems, one could imagine the unlimited number of resulting combinations for practical purposes. Examples can also include complex systems of different

non-conventional media especially ionic liquids/supercritical fluids to combine the benefits they propose (**Hobbs** et al. 2007; **Lozano** et al., 2003; **Lozano** et al., 2004).

The main focus of this thesis is on one-phase cosolvent systems (water/water-miscible organic solvents and water/water-miscible ionic liquids). Moreover, certain aspects of enzyme catalysis in water free environment (pure organic solvents and supercritical carbon dioxide) were also investigated.

I.2.1. Types of non-conventional media

I.2.1.1. Organic solvents

The discovery that enzymes can work in non-aqueous media is not new. **Sym** (1933) published his work on enzyme catalysis in organic solvents but it was left without attention for almost 50 years for an obvious reason – the lack of potential application until 1980s, when the demand for novel and enantiopure substances became significant (**Klibanov**, 2000).

The application of biocatalysis in organic solvents has many benefits (**Klibanov**, 2001; **Castro** and **Knubovets**, 2003; **Serdakowski** and **Dordick**, 2007):

a) Increased solubility of non-polar substrates and products;

b) Ability to catalyze reactions in reverse direction as compared to that in aqueous media;

c) Suppressed microbial growth;

d) Easier recovery of the enzyme and the product;

e) Potential to catalyze reactions which are impossible in water;

f) Lack of hydrolytic side reaction in contrast to aqueous media;

g) High efficiency of biocatalysts in water-organic mixtures;

h) Possible control of enzyme activity, (thermo) stability, substrate specificity and enantioselectivity by manipulating the microenvironment of the enzyme through changing the solvent.

Using enzymes in media containing little or no water greatly expands their potential as biocatalysts although the activity in many cases may decrease, as some water is essential for the maintenance of their native structure and function (**Serdakowski** and **Dordick**, 2007). Consequently, the enzyme stability is very often better in organic solvents compared to aqueous media (**Gupta**, 1992).

The effect of organic solvents on the enzyme specificity has been a subject of investigation since 1980s, when Klibanov and co-workers observed that organic solvents may severely influence the substrate specificity (**Zaks** and **Klibanov**, 1986). Studies on lipases and proteases have shown that the addition of organic solvents can drastically affect also the enantioselectivity probably because the solvents affect the flexibility of the enzyme molecule and the active site in particular (**Tawaki** et al., 1992; **Carrea** et al., 1995; **Cowan**, 1997). More recently, **Castro and Knubowets** (2003) using FTIR analysis, have evidenced that DMSO acts as enantioselectivity enhancer by deforming the molecule of subtilisin.

As the low enzyme activity in organic solvents is the main disadvantage using such systems, various techniques have been developed to create highly active enzymes. For instance, pretreatment of penicillin amidase with cesium acetate resulted in a 35,000-fold more active enzyme in hexane compared to a salt-free preparation (**Serdakowski** and **Dordick**, 2007). Other commonly used methods are the addition of detergents or lyoprotectants in the reaction mixture (**Garza-Ramos** et al. 1992), medium and reaction engineering, enzyme immobilization and protein design (**Krishna**, 2002).

I.2.1.2. Ionic liquids

Ionic liquids (ILs) are organic salts with melting points below 100°C and sometimes as low as -96°C, thus being liquid at room temperature (**Seddon**, 2000). These liquid salts exhibit excellent characteristics such as ability to dissolve polar and non-polar compounds and to show negligible vapour pressure allowing easier purification of volatile products.

In enzymology, the use of ILs instead of organic solvents has recently gained much attention. Many enzymes (mainly proteases and lipases) demonstrate better stability, activity, and enantioselectivity in ionic liquids than in organic solvents. **Kaar** et al. (2003) reported

that the application of the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM).(PF_6) instead of hexane in a two-phase system improved the initial reaction rate of a lipase 1.5-fold; **Pilissao** et al. (2006) used a mixture of ionic liquids and organic solvent to improve the enantioselectivity of a lipase and **De Diego** et al. (2004) reported that 1-ethyl-3-methyl imidazolium bis[(trifluoromethyl)sulfonyl] amide (EMIM).(NTf_2) can improve the thermostability of α-chymotrypsin via formation of a more flexible and compact 3D-structure of the enzyme molecule. A case of one-step renaturation of lysozyme by the ionic liquid ethylammonium nitrate (EAN) was reported by **Summers** and **Flowers**, 2000.

Zhao et al. (2006[b]) described an increased protease stability in 0.7 M butylpyridinium trifluoroacetate (BuPy).(CF_3COO^-) and 0.7 M 1-ethyl-3-methylimidazolium trifluoroacetate (EMIM).(CF_3COO^-). The authors tested the stability of several enzymes in different concentrations of various ionic liquids, demonstrating that their stabilizing effect follows the Hofmeister series[1] of ions (**Hofmeister**, 1888), i.e. strong kosmotropic anions (PO_4^{3-}, SO_4^{2-}, CH_3COO^-) and strong chaotropic cations (K^+, Na^+) stabilized the enzyme molecule. Based on their own and other researchers' data, **Zhao** et al. (2006[a]) set up a lyotropic series of some of the most frequently used ions in ionic liquids. The ability of cations to stabilize the enzyme generally decreases in the following order: $EMIM^+ > BuPy^+ > BMIM^+ > EtPy^+$ and the stabilizing effect of anions decreases as follows: $CH_3COO^- >$

[1] The Hofmeister series originates from the ranking of ions toward their ability to precipitate a mixture of egg proteins (**Hofmeister**, 1888). Anions appear to have a larger effect than cations, and are usually ordered as follows:

(cosmotropic/stabilizing) $SO_4^{2-} > HPO_4^{2-} >$ acetate $^- > Cl^- > NO_3^-$ (chaotropic/destabilizing)

The order of cations is given as:

(chaotropic/stabilizing) $Mg^{2+} > Li^+ > Na^+ = K^+ > NH_4^+$ (kosmotropic/destabilizing)

Small or multiple-charged ions are *kosmotropes* (or *order makers*) because they strongly order water molecules. Kosmotropes form stronger interactions with water molecules than water with itself. By contrast, *chaotropes* (or *disorder makers*) form weaker interactions with water than water with itself. An optimal stabilization of protein is achieved by salts containing kosmotropic anions and chaotropic cations which are referred to as stabilizing ions, and *vice versa* – chaotropic anions and kosmotropic cations destabilize the proteins (**Broering** and **Bommarius**, 2005; **Zhao** et al. 2006[c]).

CF_3COO^- > Cl^- > Br^- > BF_4^-. Exceptions to this rule are often due to impurities (**Davies et** al., 2004; **Lee** et al., 2006).

I.2.1.3. Supercritical fluids

Supercritical fluids are fluids at temperature and pressure above their respective critical values (**Celebi** et al., 2007). The unique combination of gas-like and liquid-like properties makes supercritical fluids useful solvents for various applications, e. g. extraction, chromatography, polymer coating, particle production, drying of biological specimens, chemical and enzymatic reactions (**Matsuda** et al., 2004; **Williams** et al., 2002).

The first report on enzymatic reactions under supercritical conditions was published in 1985 (**Hammond** et al., 1985). The authors presented a case of catalytically active polyphenol oxidase in supercritical carbon dioxide and fluoroform. Ever since, the investigation and the use of enzymatic reactions in supercritical fluids experience a rapid development (**Hobbs** and **Thomas**, 2007). The carbon dioxide is preferred among all the possible compounds because it is non-toxic, safe, inexpensive, and has appropriate for the enzymatic catalysis critical temperature and pressure. However, the application of other supercritical fluids such as methane, ethane, ethene, propane, trifluoromethane, and sulfur tetrafluoride is also increasing rapidly (**Karmee** et al., 2008).

The main reasons to use supercritical fluids as reaction media are the improved mass transfer (gas-like property), improved solubility (liquid-like property) and simple separation of the products. Furthermore, it is possible to tune enzyme activity and enantioselectivity by changing the pressure, the temperature, the water content or the solvent (**Kamat** et al., 1993). As a rule, decrease of the temperature or/and increase of the pressure lead to a decrease of the enzyme turnover (**Rezaei** et al., 2007). However, the effect of temperature and pressure on the enantioselectivity is more complex. High temperatures may cause a decrease of the enantiomeric excess because of the lower stability under these conditions. Moreover, at very high pressures the conformation of the enzyme may change by formation of carbamates from the carbon dioxide and the surface amine groups (**Celebi** et al., 2007; **Hartmann**, 2001; **Ikushima** et al., 1995).

All these effects are strongly enzyme-dependent (**Kamat** et al., 1993). For instance, **Fontes** et al. (1998) reported a case of cutinase, which enantioselectivity was not affected by pressure up to 300 bar. However, the enantioselectivity of a lipase at 55°C decreased in increasing pressure (**Matsuda** et al., 2004), whereas other lipases were only slightly (**Mase** et al., 2003) or not at all (**Rantakyla** and **Aaltonen**, 1994) affected by the pressure.

I.2.2. Important factors in non-conventional biocatalysis

I.2.2.1. Log P-value

Comparing enzyme performance in different solvents shows that one of the key factors in the non-conventional biocatalysis is the partitioning of hydration water between the bulk solvent and the enzyme. In water-miscible solvents the hydration water is more easily stripped from the enzyme molecule than in water-immiscible solvents. More polar solvent molecules can easily replace the weakly bound water and penetrate into the interior of the enzyme molecule thus causing inactivation. In contrast, non-polar solvents provide comparatively mild conditions for the enzyme because they cannot easily replace bound water, so the enzyme remains intact (**Yang** et al., 2004; **Gorman** and **Dordick**, 1992; **Serdakowski** and **Dordick**, 2007).

Therefore, it is not surprising that many authors report higher activity in non-polar solvents in comparison to polar solvents. A good correlation has been shown between enzyme properties (stability and activity) and the log P of the respective solvent. The ***log P-value*** (natural logarithm of the partition coefficient of a substance distributed between water and *n*-octanol) shows better correlation with enzyme parameters compared to the dielectric constant and the dipole moment. Now it is generally accepted that solvents with log P below 2 (good water-miscibility) are not suitable for enzymatic reaction systems, though solvents with log P above 4 (water-immiscible) provide best conditions for enzymatic catalysis (**Gupta**, 1992).

Miroliaei and **Nemat-Gorgani** (2002) reported an increase of thermostability of two alcohol dehydrogenases with increasing log P of the applied organic solvent. However, such a correlation is mainly applicable for pure solvents (**Filho** et al., 2003). Moreover, the log P criterion as a method for predicting the effect on enzyme performance can be used

only for solvents with the same functionality, e.g. alcohols and polyols (**Khmelnitsky** et al, 1991).

I.2.2.2. Water activity

A further important parameter for the enzyme activity and stability in almost water-free systems is the water activity (**Gupta** and **Roy**, 2004). The importance of considering the effect of water activity has been first studied in food industry. It was found that not the water content but the available "free" water is the factor that leads to food spoilage (**Andersson**, 1980).

Water content is the total amount of water in a substance. In contrast, ***water activity*** (a_w) is technically defined as the ratio of the vapour pressure of any chemical substance to the vapour pressure of pure water (a_w=1.0 or 100%) at the same temperature. In other words, water activity is the amount of "free" water available in a substance that is not chemically bound. The "free" water, for instance, would be available to support the growth of microorganisms. All microorganisms have optimum and minimum a_w requirements. Bacteria usually grow in environment with a high a_w, while yeasts and moulds may grow at lower water activities. Most microorganisms do not grow in environment with a_w below 0.60 (**Grant**, 2004).

Most investigations on the effect of water activity on the enzyme performance are carried out with hydrolases, e.g. lipases and proteases, where water participates in the reaction. Many enzymes require a_w below 1, with enzyme activity peaking at $a_w \sim 0.8$. Lower water activity will usually cause decrease of the enzyme activity since it is generally considered that at low water activity the enzyme is inadequately hydrated, which causes enzyme aggregation (**Valivety** et al., 1992[b]).

Water activity is an important factor for the enzyme stability (**Lemos** et al., 2001). Although the effect of increasing ***temperature*** on enzymes follows the same trend in non-conventional media as in water (higher activity causes faster inactivation), the enzyme molecule is more rigid and generally exhibits higher stability in non-conventional media due to reduced water activity (**Volkin** et al., 1991, **Gupta**, 1992). Moreover, enzymes that are generally more stable in water also tend to show higher stability in non-conventional media (**Rupley** and **Careri**, 1991).

Water activity is crucial for reactions involving water either as a substrate or a product because it affects the equilibrium of the reaction. Moreover, decreasing water activity may have different effects on enantioselectivity, which for different enzymes was found to increase, decrease or remain unchanged (**Carrea** et al., 1995; **Rariy** and **Klibanov**, 2000; **Fontes** et al. 1998).

I.2.2.3. "Enzyme memory"

In aqueous buffers, the enzyme activity is independent on the "history" of the enzymes, which is however not the case in water-free or nearly water-free media. The enzyme activity in water-free media depends strongly on the way the enzyme was treated before it got into contact with the solvent (**Ke** and **Klibanov**, 1997). The enzyme activity in water-free systems is affected mainly by the pH of the aqueous solution from which the enzyme was lyophilized (**Xu** and **Klibanov**, 1996; **Gupta** and **Roy**, 2004); therefore, this "***enzyme memory"*** is usually referred to as ***"pH-memory"***, which corresponds to the protonation state of the enzyme at the respective pH.

Furthermore, due to the lack of "molecular lubricant" water the enzyme keeps its former conformation and "remembers" it. **Dai** and **Klibanov** (1999) demonstrated that co-lyoplilization of enzymes from aqueous solution containing organic solvents or substrates induced a conformation which resulted in better activity in water-free media, indicating that enzymes show "***solvent memory"***, too.

I.2.3. Non-conventional media to solve practical problems of applying benzaldehyde lyase and benzoylformate decarboxylase

The use of non-conventional media may be appropriate in all cases when the aqueous buffers fail to provide optimum reaction conditions.

The enzymes, which are in the focus of this dissertation, benzaldehyde lyase (BAL) and benzoylformate decarboxylase (BFD) belong to the group of thiamine-diphosphate (ThDP)-dependent enzymes and catalyze the formation of chiral 2-hydroxy ketones, which are important precursors e.g. for the pharmaceutical industry. The main problem such

processes encounter is the low solubility of the aromatic aldehydes employed as substrates in aqueous buffers. As a consequence the maximum reaction rates cannot be achieved.

Complete replacement of water with water-immiscible solvents or addition of some water-miscible solvents improves the solubility of aromatic aldehydes such as benzaldehyde and the resulting products (benzoins) (**Appendix**, **Table A3, A4**). Besides, the solubility of benzaldehyde in non-polar solvents is often > 1 M. Addition of 20-30 vol% DMSO to the reaction mixture considerably improves the solubility of aromatic aldehydes and thus the catalytic performance of the enzymes (**Stillger** et al., 2004; **Domínguez de María** et al., 2006). Although aqueous/DMSO mixtures are routinely used as reaction systems for both enzymes, the effect of the solvent has not been characterized in detail.

I.3. Benzaldehyde lyase and benzoylformate decarboxylase

I.3.1. Common characteristics

Benzaldehyde lyase and ***benzoylformate decarboxlase*** are thiamine diphosphate-dependent enzymes. The cofactor thiamine diphosphate (ThDP) (**Fig. 2**) is the active form of vitamin B_1 and is found in many enzymes which catalyse carbon-carbon bond formation and cleavage. ThDP consists of pyrimidine and thiazole ring, which in turn is connected to two diphosphate functional groups. The part of the molecule most commonly involved in catalysis is the thiazole ring, which contains nitrogen and sulphur (**Erixon** et al., 2007; **Fiedler** et al., 2002; **Frank** et al., 2007).

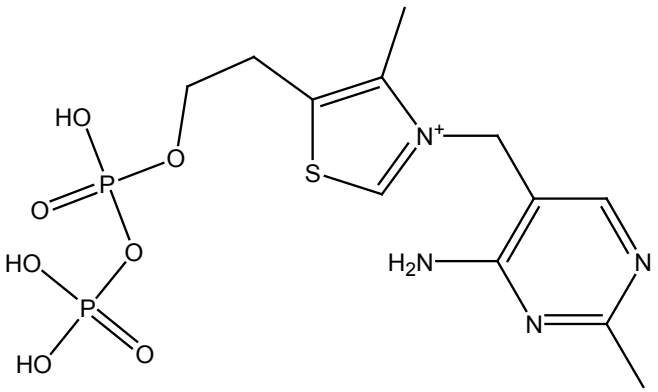


Figure 2: Thiamine diphosphate.

The ***benzaldehyde lyase*** (BAL, EC 4.1.2.38) from *Ps. fluorescens Biovar I* has been first described by **Gonzalez** and **Vicuna** (1989) as a new ThDP- and divalent cation (Mg^{2+})-dependent enzyme, able to cleave the acyloin linkage of benzoin to two molecules of benzaldehyde. As later elucidated, this reaction is highly enantioselective towards the *(R)*-enantiomer enabling kinetic resolution of racemic benzoins (**Demir** et al., 2001).

Although BAL was initially described to show only lyase activity, it was later discovered that the enzyme can also catalyse the reverse reaction producing a broad range of substituted *(R)*-benzoins (**Demir** et al., 2001, 2002; **Janzen** et al., 2006; **Domínguez de María** et al., 2006, 2007; **Kühl** et al., 2007). Moreover, the enzyme is able to catalyze the formation of highly enantiopure *(R)*-hydroxypropiophenone derivatives, using various aliphatic aldehydes as acceptor and aromatic aldehydes as donor substrates (**Demir** et al., 2002, 2003; **Dünkelmann** et al., 2002; **Domínguez de María** et al., 2006 ; **Hildebrandt** et al., 2007). However, the carboligation of aliphatic aldehydes is catalysed with only moderate enantioselectivity (**Domínguez de María** et al., 2007).

BAL has been characterized with respect to pH, temperature, buffer salts, cofactors and organic cosolvents (**Janzen** et al., 2006). For the lyase reaction the enzyme exhibits a distinct narrow activity optimum at pH 8, whereas the broader activity optimum for the ligase reaction lies between 7.5 and 8.5. The enzyme is most stable at pH-values between 6 and 8 in potassium phosphate, Tris and imidazole buffers. BAL is unstable at temperatures above 37°C and the thermal inactivation is a function of the cofactor concentration in the buffer, indicating that the loss of cofactors is one of the main reasons for the enzyme inactivation. The optimum cofactor concentrations were determined as 0.1-5 mM ThDP and 1-5 mM $MgSO_4$. The isoelectric point of recombinant BAL with a C-terminal His-Tag, which was used in this thesis, was determined at pH 4.6. It was shown that 20 vol% DMSO in potassium phosphate buffer facilitates the benzoin synthesis and is superior to phosphate buffer without DMSO or PEG-400/phosphate buffer mixture (**Janzen** et al., 2006).

Thus, BAL is routinely used in aqueous buffer containing 20-30 vol% DMSO (**Stillger** et al., 2006). However, reaction systems containing no DMSO are of great interest because this solvent causes problems during the down-stream processing of the formed 2-hydroxy ketones (**Stillger**, 2004). Therefore, attempts were made to establish better reaction systems using other media. For instance, the enzyme was tested for stability and activity in

two-phase systems (**Domínguez de María** et al., 2008), where ethers (e.g. MTBE) were found to be optimal. Same authors presented also a successful whole cell biocatalysis with BAL in MTBE/phosphate buffer system. **Mikolajek** et al. (2007) established a solid/gas system with immobilized BAL and found that the enzyme is more active and more stable at high water activity (~100 %). A BAL- catalysed synthesis of HPP derivatives in continuously operated enzyme membrane reactor (EMR) was established in order to overcome the problem with precipitation of reaction products (**Hildebrand** et al., 2007).

Benzoylformate decarboxylase (BFD) (EC 4.1.1.7) from *Pseudomonas putida* is ThDP and Mg^{2+}-dependent enzyme which was first reported by **Hegeman**, 1970. The enzyme is part of the mandelate pathway and catalyses the non-oxidative decarboxylation of benzoylformate (**Tsou** et al., **1990**). As a side reaction, BFD is able to catalyse the enantioselective synthesis of (*S*)-2-hydroxypropanone derivatives (**Wilcocks** et al., 1992; **Demir** et al., 1999; **Iding** et al., 2000; **Dünnwald** et al., 2000). This is in contract to BAL, which catalyses the synthesis of *(R)*-2-hydroxypropanone derivatives.

BFD is able to ligate a broad range of aromatic, heteroaromatic, aromatic vinyl-aldehydes (as donor substrates) preferably with acetaldehyde (as acceptor substrate). Besides, BFD shows activity with aromatic and heteroaromatic acceptor substrates producing enantiopure *(R)*-benzoin derivatives but in contrast to BAL with a very low reaction rate (**Iding** et al., 2000). Another difference to BAL is that *ortho*-substituted aldehydes (as donor substrates) are only poor substrates for wild type BFD.

The substrate range of BFD has been studied intensively. The substrate specificity was broadened using site-directed mutagenesis and directed evolution: variants able to accept *ortho*-substituted aldehydes with improved activity in organic cosolvents (**Lingen** et al., 2002, 2003) and variants with improved benzoin-forming activity (**Dünkelmann** et al., 2002; **Pohl** et al., 2002) were generated.

BFD was characterized with respect to pH, temperature and cofactor binding (**Iding** et al., 2000). The optimum pH for the decarboxylase activity is between 5.5 and 7.0 with a distinct maximum at pH 6.2. However, the enzyme is most stable between pH 6-8. BFD is significantly more thermostable than BAL. Although it is rapidly deactivated at 80°C, BFD is stable at 60°C for 2 hour and has a half-life of 36 ± 7 d at 20°C. For optimum activity and stability the enzyme requires 0.5 mM ThDP and 0.5 mM $MgSO_4$ in potassium phosphate

buffer. The absence of the cofactor ThDP in the buffer leads to a 10-fold decrease of half-life at 20°C.

Like BAL, BFD is routinely used in DMSO/phosphate buffer systems. Wild type BFD tolerates up to 30 vol% DMSO, but in case of the His-tagged BFD only 10 vol% DMSO are applicable (**Dünnwald** et al., 2000). To improve the enzymatic performance, various reaction systems were explored. For instance, whole cell biocatalysis with BFD in two-phase system (MTBE/ phosphate buffer) was reported by **Domínguez de María** et al. (2008). Moreover, many BFD variants from a saturation mutagenesis library were tested and expressed better activity in 1.5 M ethanol and 20 vol% DMSO than in buffer. The enantioselectivity of some variants was slightly affected by the solvents (**Lingen** et al., 2002). **Mikolajek** et al. (2007) established a solid/gas process with immobilised BFD in which the enzyme showed better stability and worse activity than BAL under the same conditions. Besides, the optimum water activity was significantly lower compared to BAL (~50 %).

Many BFD variants have been created to investigate the role of certain amino acid side chain residues in the active site and to improve stability, carboligase activity and substrate specificity. The variant BFD*H281A* is of particular interest, as it was identified as a potent catalyst for asymmetric cross-carboligation (**Dünkelmann** et al., 2002; **Pohl**, 2002). BFD*H281A* is the most active BFD variant with respect to *(R)*-benzoin condensation. The substitution of the histidine from the active site by alanine provides more space for acceptation of aromatic aldehydes and improves the carboligase activity, thus making it a more BAL-like enzyme (**Knoll** et al., 2006), which is the reason for choosing this particular variant together with the wt BAL for this research.

I.3.2. Different main reactions

The main differences between both enzymes can be illustrated with the following model reactions (**Table 1**), (**Pohl**, 2002):

With BAL as a catalyst, the formation of *(R)*-2-HPP starting with a mixture of benzaldehyde and acetaldehyde (acetaldehyde in excess) proceeds via a benzoin intermediate which further reacts with acetaldehyde to *(R)*-2-HPP with *ee* > 95% in presence of DMSO

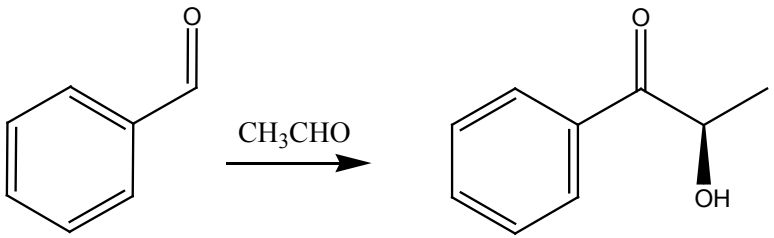
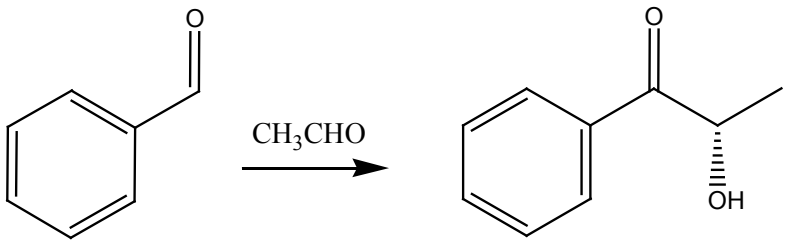
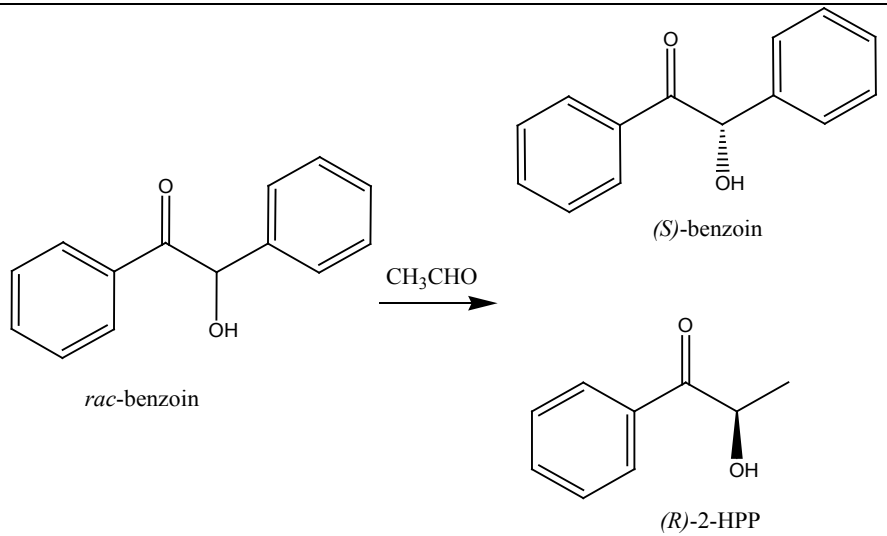
(**Stillger,** 2004; **Stillger** et al., 2006). With BFD, the *(S)*-enantiomer is formed with *ee* = 92% (**Iding** et al., 2000; **Pohl** et al., 2002). The formation of *(R)*-2-HPP catalysed by BAL is also possible starting with benzoin and acetaldehyde, cleaving only the *(R)*-benzoin out of the racemic mixture (**Demir** et al., 2001).

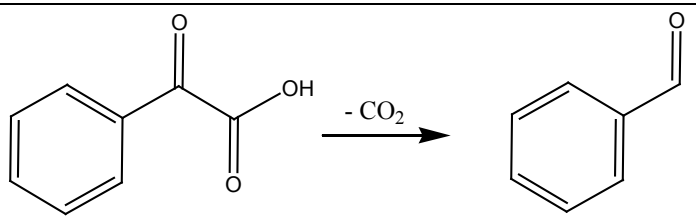
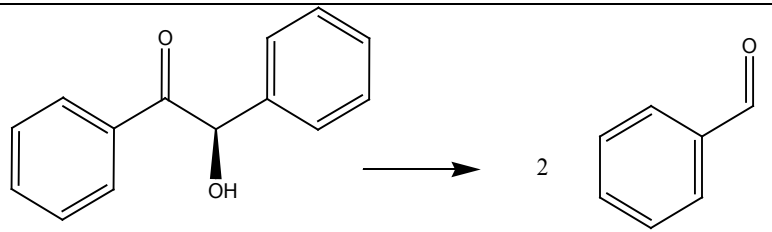
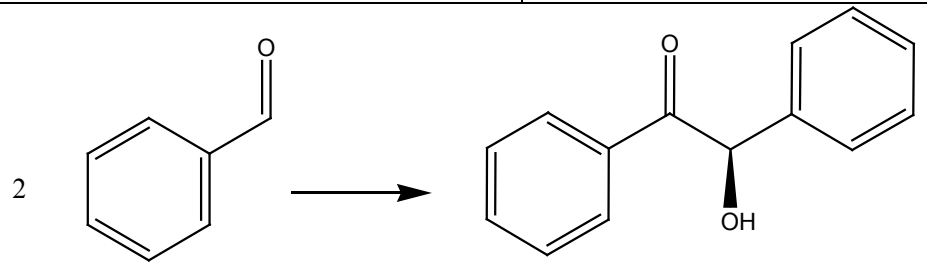
The benzoin cleavage is a specific reaction for BAL and is not observed with BFD or any other known ThDP-dependent enzyme. In contrast, the decarboxylase activity is typical for BFD and was not observed with BAL.

I.3.3. Common side reaction – the benzoin synthesis

Both enzymes catalyse a common reaction – the benzoin synthesis. The formation of (*R*)-benzoin is catalyzed by both enzymes with excellent enantioselectivity (more than 99%) and high yield (97%, BAL and 70%, BFD) in presence of DMSO (**Pohl** et al., 2002). The catalytic cycle of the reaction catalysed by BAL (**Demir** et al., 2001) can be subdivided into three main steps (**Fig. 3**). At first, the reactive ylide form of ThDP binds a benzaldehyde molecule to form the first covalent intermediate: hydroxybenzyl-ThDP (HBz-ThDP) (step 1). After deprotonation of C2α, a highly reactive nucleophilic enamine-carbanion intermediate is formed, which adds to the second benzaldehyde molecule to yield benzoin-ThDP (step 2). Finally, (*R*)-benzoin is released from that adduct and the cofactor is regenerated (step 3). The protonation (and isomerization) steps can be considered to occur very fast. The same mechanism is used to describe the reaction with BFD but only in the direction of synthesis, because the benzoin cleavage was not yet observed with this enzyme.

Table 1: Typical reactions of benzaldehyde lyase and benzoylformate decarboxylase.

Reaction	BAL	BFD
Formation of 2-HPP form benzaldehyde and acetaldehyde	 benzaldehyde *(R)*-2-HPP	 benzaldehyde *(S)*-2-HPP
Formation of 2-HPP from benzoin and acetaldehyde	 *rac*-benzoin *(S)*-benzoin *(R)*-2-HPP	No reaction

Reaction	BAL	BFD
Decarboxylation of benzoylformate	No reaction	 benzoylformate — benzaldehyde
Benzoin cleavage	 *(R)*-benzoin — 2 benzaldehyde	No reaction
Benzoin synthesis	 2 benzaldehyde — *(R)*-benzoin	

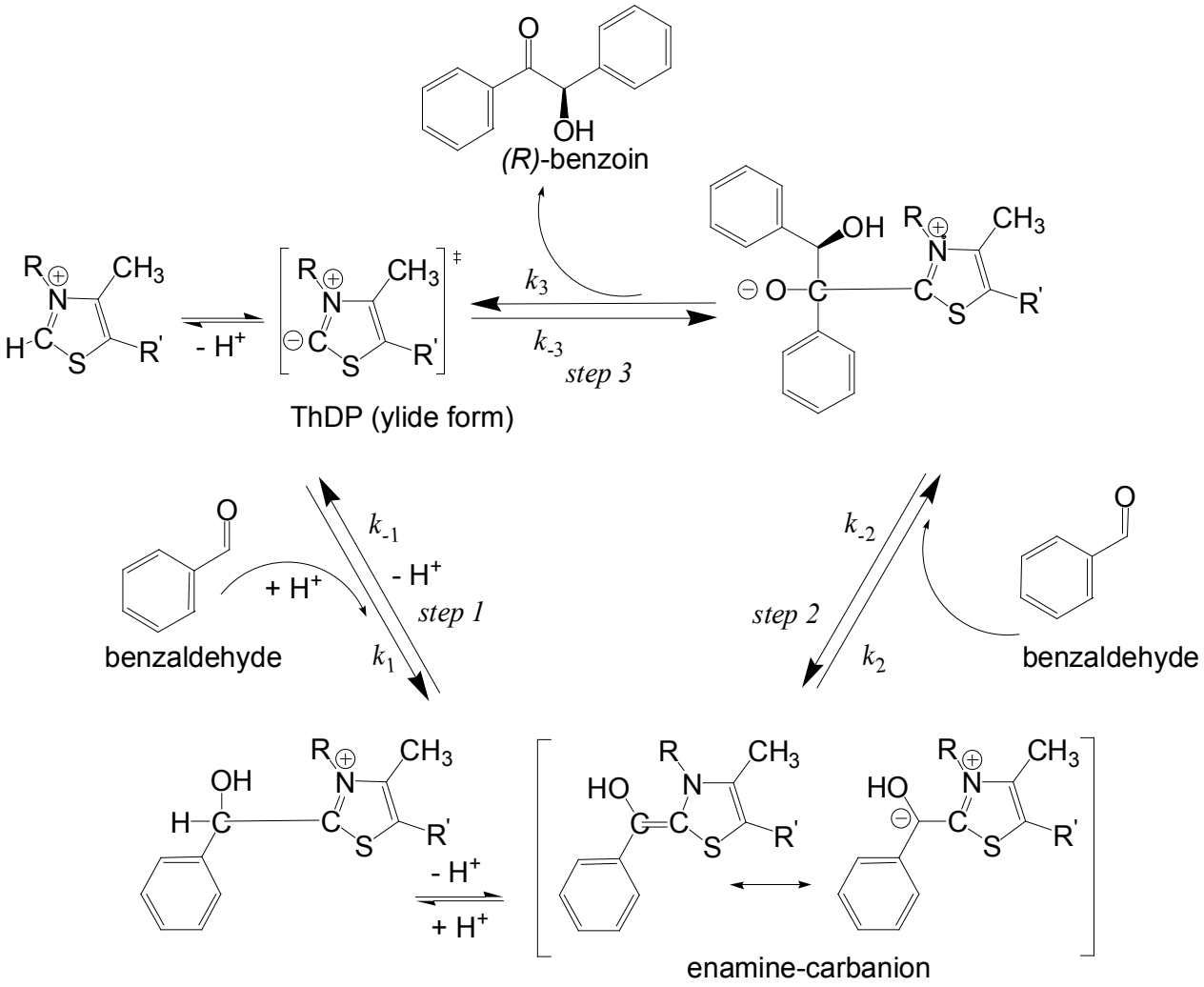


Figure 3: Mechanism of BAL-catalyzed benzoin formation/cleavage. The mechanism holds for BFD only in the forward reaction (benzoin formation).

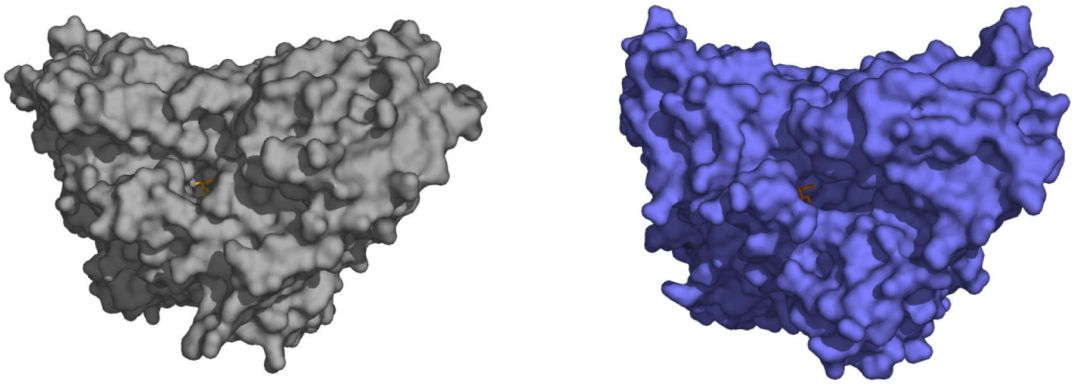
I.3.4. Mechanistic kinetic model for the benzoin synthesis

So far, the donor-acceptor concept has been considered only for two different substrates. By varying the concentration of one respective substrate, their Michaelis constants (K_{mA} and K_{mB}) can be determined. In case of the benzoin synthesis, where the same substrate is donor and acceptor, so far only one apparent K_m value has been assumed (**Stillger** et al., 2006; **Hildebrand** et al., 2007).

Recently, a ***mechanistic kinetic model*** has been derived which can estimate kinetic parameters using progress curve analysis by monitoring the substrate decrease and the product increase over time. The model can estimate the maximum turnover number for the forward reaction k_{catf}, the thermodynamic equilibrium constant K_{eq} for the overall reaction and the Michaelis constants for the donor and the acceptor, even in case they are identical (K_{mA} and K_{mB}). Moreover, dependent parameters can be calculated using the equations given in **Chapter II.5** and the microscopic parameters for every reaction step (k_1, k_{-1}, k_2, k_{-2}, k_3 and k_{-3}) (**Fig. 3**) can be calculated with a very good accuracy (**Zavrel** et al., 2008).

I.3.5. Structural comparison of BAL and BFD

Although the sequence similarity of BAL (**Hinrichsen** et al., 1994) and BFD (**Tsou** et al., 1990) is low, their three-dimensional structures are highly similar (**Hasson** et al., 1998; **Polovnikova** et al, 2003; **Mosbacher** et al., 2005), (**Fig. 4**).



BAL BFD

Figure 4: BAL and BFD dimers: surface representation. One active site is present at the bottom of a channel indicated by bound ThDP (orange). The picture was produced by Dr. Michael Knoll, University of Stuttgart, using the program Pymol (DeLano, 2002).

Both enzymes are homotetramers, with each momomer consisting of three domains: α, β and γ. The molecules can be described as dimers of dimers and each dimer contains two active sites situated in the contact areas between two monomers.

BAL and BFD show very similar binding modes for ThDP. In both cases ThDP adopts a V-conformation and is fixed by identical binding motives. The diphosphate groups of the cofactor are tightly bound to the polypeptide of the γ-domain via Mg^{2+}.

Moreover, the active site is more spacious in BAL whereas the entrance to the active site is wider in BFD (**Fig. 4**). The latter is due to a C-terminal helix which covers the entrance of the active site of BAL but is absent in BFD (**Fig. 5**). The substitution of the histidine by alanine in case of BFD*H281A* provides more space for the access of aromatic aldehydes to the active site, which is assumed to explain the higher reaction rate of the benzoin condensation with this variant relative to the wild type enzyme (**Fig. 5**).

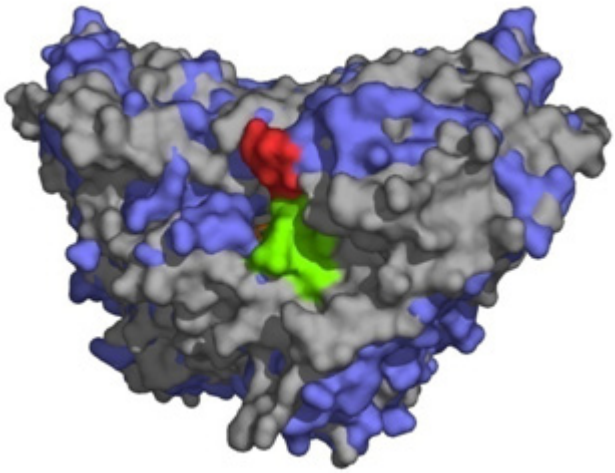


Figure 5: Superimposition of BAL and BFD dimers. The C-terminal helix of BAL (green) and His281 in BFD (red) is highlighted. The picture was produced by Dr. Michael Knoll, University of Stuttgart, using the program Pymol (DeLano, 2002).

The shape of the binding site explains the different enantioselectivities of BFD and BAL with respect to the formation of 2-hydroxypropiophenone. BAL is always *(R)*-selective and can accept longer aliphatic aldehydes, whereas the enantioselectivity of BFD is a function of the size of the acceptor aldehyde (**Knoll** at al., 2006). Besides, in case of formation of benzoin both enzymes are (*R*)-selective, with only BAL being able to cleave (*R*)-benzoin with a measureable rate. This difference is a consequence of the different sizes of the active sites (**Fig. 6**). The modelling studies showed that *(R)*-benzoin can fit easily in the active site but any (*S*)-enantiomers (benzoin or *(S)*-2-HPP) experiences severe sterical hindrance.

To explain the *(S)*-selectivity of BFD, **Knoll** et al. (2006) proposed an active site model based on structural studies. The authors identified a small *S*-pocket (**Fig. 6**, **BFD**) in which only small alphatic aldehydes can fit as donor substrates. Benzaldehyde cannot fit into the *S*-pocket; therefore it adopts such a position that allows only the formation of *(R)*-

benzoin. To verify the model, several mutants in the *S*-pockets were investigated (**Gocke** et al., 2008).

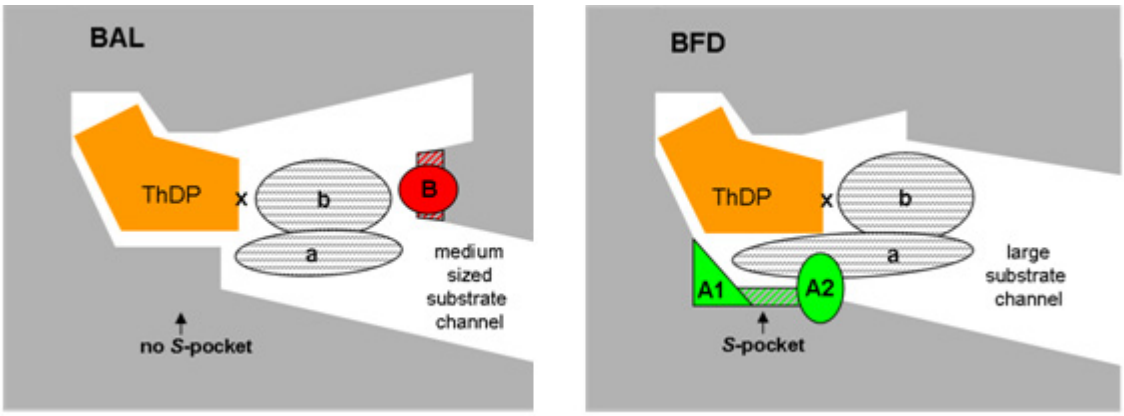


Figure 6: Schematic presentation of BAL and BFD active sites. The cofactor ThDP is bound to the active site in a V-conformation; X: C2-Atom of the thiazolium ring; A: Amino acids forming the *S*-pocket (A1: Moiety with significant influence on the size of the *S*-pocket, A2: Moiety defining the entrance to the *S*-pocket); a: Acceptor aldehyde binding site; b: Binding site for 2-keto acids or donor aldehydes, B: Moiety influencing the donor aldehyde binding site (from: Gocke et al., 2008).

I.4. Goals

The focus of this thesis is the comparative characterization of the enzymes BAL and BFD*H281A* in one-phase aqueous/organic and aqueous/ionic liquid systems, as well as water-free systems, such as pure organic solvents and supercritical CO_2. The main problem of the reactions catalyzed by these enzymes in aqueous buffer is the low solubility of the substrates and products, which can be overcome by addition or complete replacement of the water by appropriate solvents. However, their effect on the enzymes needs to be investigated in detail.

The characterization includes:

- **Determination of the influence of various non-conventional media on the enzyme activity using initial rate measurements.** In order to compare the enzymes on the same basis, a common model reaction for both enzymes - the synthesis of benzoin from benzaldehyde - was chosen. To elucidate the solvent effects, additional experiments on the enzyme solubility (e.g. salting-out effect) and

the phenomena related to pH-changes (activity in different pH) had to be conducted.

- **Investigation of the enzyme (thermo) stability in non-conventional media.** In addition to the stability at 30°C (standard reaction conditions), it was important to determine also the stability at elevated temperatures.

- **Studying the structural changes of the enzyme molecule caused by different non-conventional media.** To investigate the unfolding of the enzymes in the presence of non-conventional media, spectroscopic methods, such as fluorescence- and circular dichroism spectroscopy were chosen. Together with the respective activity data from the initial rate measurements, these studies provide important information concerning the enzymatic structure-function relationship.

- **Investigation of the cofactor stability and binding.** The cofactor ThDP is non-covalently bound to the active sites and is an important part of the reaction system. Therefore, by studying its performance in various solvents, certain aspects of the enzymatic stability and activity could be explained.

- **Effect of non-conventional media on the enantioselectivity.** Potential effects of non-conventional media on the enantioselectivity were studied using two carboligation reactions: the benzoin and the 2-HPP synthesis. The results can be used to optimize the reaction conditions and to identify potential structural changes, as the enantioselectivity depends on the active site geometry.

- **Studies of the reaction kinetics and mechanism of benzoin synthesis in aqueous buffer and in the presence of selected cosolvents.** To completely understand the influence of organic solvents on the enzyme activity, a mechanistic kinetic model was applied to determine the kinetic parameters and micro-reaction constants. The detection of reaction intermediates by ^{1}H NMR was used as a qualitative method to verify the results obtained with the mechanistic kinetic model.

II. MATERIALS AND METHODS

II.1. Materials

II.1.1. Chemicals

All reagents (aldehydes, benzoin and 2-HPP) and organic solvents were purchased from Roth; deuterated acetone and DMSO were from Sigma Aldrich; ionic liquids were form Solvent Innovation and the buffer salts from Roth. All chemicals were analytically pure.

II.1.2. Enzymes

Expression of wt BAL and BFD*H281A* was performed using the recombinant *E. coli* strain SG13009 and previously created vectors pBALHis (**Janzen** et al., 2006) and pBFD*H281A*His, respectively (**Siegert**, 2000). Both enzymes were purified to homogeneity in two steps: Ni-NTA affinity chromatography and desalting gel-filtration (see **II.4.1**).

II.1.3. Cultivation media

For the cultivation of the recombinant *E. coli* strains, liquid and solid Luria-Bertani (LB) media were used. 1 L liquid LB medium contained 10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract. The solid LB medium contained additionally 15 g/l agar-agar. The resulting solutions were autoclaved for 20 min at 121°C. The liquid LB medium was kept at room temperature. After cooling down to 50°C, 0.1 mg/ml antibiotics (kanamycin and ampicillin) were added to the medium containing agar-agar. Immediately afterwards, the mixture was poured into Petri dishes each with 25 ml and kept at 4°C.

II.2. Microbiological methods

II.2.1. Cultivation of BAL and BFD*H281A* in a shake flask

For cultivating *E. coli* in a shake flask first a pre-culture was prepared. 100 ml pre-culture consisted of 100 ml LB medium containing 0.1 mg/ml antibiotics (kanamycin and ampicillin) and one colony inoculum. The pre-culture was incubated at 30°C and 120 rpm

for 5 hours or overnight and was used as inoculum for cultivation in a larger scale: 1-2 L in a shake flask. At OD_{600}=0.6 the culture was induced with 1 mM IPTG (100 mM stock solution in 70 vol% ethanol) and harvested after 24 hours.

II.2.2. Cultivation of BFD*H281A* in a fermenter

High cell density fed-batch cultivation (**Korz** et al., 1995) was performed in a 20 L fermenter from Pierre Guerin Technologies. The fermenter was equipped with monitors and controls for pH, temperature and oxygen saturation. Stirring speed was 800 rpm and air supply was controlled to maintain app. 100% saturation. The pH was maintained within range of 6.9-7.1 by automatic addition of 5 M HCl and 5 M KOH. The batch medium (10 L) consisting of yeast extract, salts, glucose, trace elements and anti-foam was autoclaved in the fermenter. After cooling down, the vitamins, thiamine, antibiotics and 100 ml inoculum were added. Samples were taken during fermentation to monitor bacterial growth, utilisation of substrate and enzyme expression. The feed (5 L total) was started when the batch glucose was exhausted. After 24 hours, the culture was induced with 1 mM IPTG and harvested after further 32 hours. For composition of stock solutions, batch and feed media see **Appendix** (**Tables A1, A2**).

II.2.3. Cell harvest

The cells were harvested by centrifugation for 30 min at 10,000 rpm and 4°C using Sorvall RC-5B Centrifuge, DuPont. Thereafter, the cells were immediately subjected to ultrasonic disintegration or stored at -20°C.

II.2.4. Cell desintegration

Frozen or freshly harvested cells (30 g) were dissolved in 50 mM potassium phosphate buffer, (pH 7, 4°C) and homogenized on ice for 4 x 5 min with 70% amplitude and 0.5 cycle using a sonicator UP 200s from Dr. Hielscher GmbH, Germany. Homogenates were centrifuged for 30 min with 15,000 rpm, at 4°C and the supernatant containing the enzyme was kept at 4°C until purification.

II.3. Molecular biology methods

II.3.1. Transformation of chemically competent *E. coli* cells

A vial of chemically competent cells – *E. coli* SG 13009, kanamycin resistant – was slowly thawed on ice. Thereafter, 100 µl of the bacterial suspension was transferred into another vial, mixed with 1 µl of plasmid solution (~10 ng plasmid-DNA) and incubated for 30 min on ice. The sample was heated in a water bath at 42°C for exactly 1 min and immediately transferred on ice for 3 min. Afterwards 400 µl of liquid LB medium was added and the vial was incubated at 37°C with shaking at 100 rpm. After 120 min, 100 µl of bacterial suspension were transferred on an agar plate. The residual suspension was centrifuged for 3 min at 6,000 rpm, 300 µl of supernatant discarded, the pellet resuspended in the remaining medium and the whole mixture plated on another agar plate. Both agar plates were incubated for 24 hours at 37°C.

II.4. Protein chemistry methods

II.4.1. Enzyme purification

The enzymes, expressed as C-terminal hexahistidine fusion proteins, were purified to ~ 95% homogeneity in two chromatographic steps.

After washing the system with equilibration buffer (50 mM potassium phosphate buffer) for 2 hours, the supernatant (from **II.2.4**) was loaded on a chromatographic column (50 ml) packed with Ni-NTA agarose resin Superflow (Qiagen). Both enzymes were eluted by shifting the washing buffer (50 mM potassium phosphate buffer containing 50 mM imidazole) to elution buffer (50 mM potassium phosphate buffer containing 350 mM imidazole). The flow rate was set to 3 ml/min. The protein was subsequently desalted using a size-exclusion chromatographic column (1 L) packed with Sephadex G25 from GE Healthcare. The mobile phase was 10 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.1 mM ThDP at flow rate 10 ml/min. Both chromatographic steps were performed using an ÄKTA purifier (GE Healthcare). All used buffers had pH 6.5 for BFD*H281A* and pH 7 for BAL. During purification, BAL activity was measured using the benzoin cleavage assay (**II.4.4.1**) and BFD*H281A* activity was measured using the benzoylformate decarboxylation assay (**II.4.4.2**). After purification, the enzymes were stored freeze-dried.

II.4.2. Protein determination

The protein content was determined using the Bradford method (**Bradford**, 1976). The standard curve was prepared using BSA and it showed a linear correlation between the absorbance at 595 nm and the protein concentration in the range from 10 to 100 µg ml^{-1}.

To measure the soluble protein in presence of solvents, the samples were incubated for 1 hour in the respective solvent concentrations and subsequently centrifuged at 13,000 rpm for 5 min to remove the precipitated protein. The absorbance of the protein samples was corrected by subtracting the background absorbance of the solvents omitting the protein.

II.4.3. SDS-PAGE

SDS-PAGE was used to determine the enzyme purity and expression. The composition of the casting gel (12%) and the stacking gel (5%) is given in **Table 2**. As marker proteins, prestained molecular weight standards from BioRad Laboratories were used.

Table 2: Ingredients for gel solutions (for two gels).

Chemic	*Stacking gel*	*Separation gel*
Acryla	1.35 ml	6 ml
1.5 M	---	3.75 ml
0.5 M	1.88 ml	---
MilliQ	4.16 ml	5.03 ml
10%	75 µl	150 µl
10%	75 µl	150 µl
TEME	15 µl	15 µl

II.4.4. Determination of enzyme activity. Activity assays

II.4.4.1. Benzoin cleavage assay (BAL only)

The reaction mixture consisted of 700 µl 50 mM potassium phosphate buffer (pH 8) containing 2.5 mM $MgSO_4$, 0.5 mM ThDP, 150 µl substrate solution (15 mM benzoin pre-dissolved in PEG 400), 50 µg 7 mM NADH and 50 µl 5.5 mg/ml horse liver alcohol dehydrogenase (HL-ADH). After 5 min of preincubation at 30°C the reaction was started by addition of 50 µl BAL solution. The decay of NADH (ε = 6.3 L $mmol^{-1}$ cm^{-1}) was

followed at 340 nm for 60 s using Beckman Coulter, DU 650 spectrophotometer. Activity was calculated using the following equation (**Janzen**, 2002):

$$\frac{U}{ml} = \frac{\Delta E}{\min} \cdot \frac{V}{v \cdot d \cdot \varepsilon} = \frac{\Delta E}{\min} \cdot \frac{1}{0.05 \cdot 1 \cdot 6.3} : 2 = \frac{\Delta E}{\min} \cdot 1.59 \qquad \textbf{(eq. 1)}$$

U	unit (µmol/min)
ΔE/min	change of extinction
V	total volume (ml)
v	enzyme volume (ml)
d	optical pathway (cm)
ε	extinction coefficient (L mmol^{-1} cm^{-1})

The photometric coefficient ($\frac{V}{v \cdot d \cdot \varepsilon}$) was divided in 2, as one molecule of benzoin gives two molecules of benzaldehyde. Due to contamination of the benzoin with benzaldehyde, the background activity of samples without the enzyme was measured and subtracted from the activity with enzymes.

II.4.4.2. Benzoylformate decarboxylase assay (BFD*H281A* only)

The reaction mixture consisted of assay buffer (700 µl 50 mM potassium phosphate buffer (pH 6.5) containing 2.5 mM $MgSO_4$, 0.5 mM ThDP), 100 µl substrate solution (50 mM benzoylformate in assay buffer), 100 µl 2.5 mM NADH and 50 µl 5.5 mg/ml HL-ADH. After 5 min of preincubation at 30°C the reaction was started by addition of 50 µl BFD*H281A* solution. The decay of NADH was followed at 340 nm for 60 s using Beckman Coulter, DU 650 spectrophotometer. Activity was calculated using **eq. 1**, as follows (**Janzen**, 2002):

$$\frac{U}{ml} = \frac{\Delta E}{\min} \cdot \frac{V}{v \cdot d \cdot \varepsilon} = \frac{\Delta E}{\min} \cdot \frac{1}{0.05 \cdot 1 \cdot 6.3} = \frac{\Delta E}{\min} \cdot 3.17$$

II.4.4.3. 3,3´,5,5´-tetramethoxy-benzoin formation assay

The fluorometric assay was used to determine the transformation of DMBA (3,5-dimethoxybenzaldehyde) to *(R)*-3,3´,5,5´-tetramethoxy-benzoin (TMB) (**Zavrel** et al., 2008). The assay was performed at 30°C using a spectroflourometer LS50B from PerkinElmer. The reaction mixture contained 50 mM phosphate buffer (pH 8 for BAL and 6.5 for BFDH*281A*) with various cofactor concentrations and 3 mM DMBA. The reaction

was started by addition of 20 µg/ml BAL or 60 µg/ml BFD*H281A*. The DMBA concentration was monitored by excitation at 360 nm and recording the fluorescence intensity at 470 nm. Excitation and emission slits were set to 5 and 7.5 nm, respectively. All the spectra were measured in 1 cm path Suprasil quartz cuvettes from Hellma.

Initial DMBA concentration should not exceed 3 mM due to limited product solubility.

II.4.4.4. Benzoin formation assay by HPLC

The carboligase activity was determined by measuring the initial rates of benzoin formation from benzaldehyde by a discontinuous assay using an HPLC-System from Gynkotek. The reaction mixture consisted of 20 mM benzaldehyde in potassium phosphate buffer (50 mM), containing 2.5 mM $MgSO_4$ heptahydrate, 0.5 mM ThDP and BAL (5-10 µg/ml) or BFD (40-60 µg/ml). BAL was studied at pH 8 and BFD*H281A* was studied at pH 6.5. The temperature for both enzymes was 30°C.

The method was used to test the enzyme activity in presence of different solvents: 2-propanol, ethanol, DMSO, acetone and acetonitrile, as well as the water-miscible ionic liquids Ecoeng 21M (1-ethyl-3-methylimidazolium 2-ethylsulfate), Ecoeng 1111P (1,3-dimethyl-imidazolium dimethylphosphate), 1-ethyl-3-hydoxymethylpiridinium ethylsulfate and BMIM.BF_4 (1-buthyl-3-methylimidazolium-2-tetrafluoroborate). Different amounts of solvent were mixed with 50 mM potassium phosphate buffer (pH 8 for BAL and 6.5 for BFD*H281A*) containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP in 1.5 mL Eppendorf tubes. The tubes containing the respective solvent, the enzyme solution and the substrate were preincubated in a thermomixer (Eppendorf).

To measure the amount of benzoin formed, 50 µl samples were withdrawn at appropriate time intervals, diluted with 950 µl acetonitrile (1:20) to inactivate the enzyme, centrifuged at 13,000 rpm for 5 min and analyzed on HPLC instrument from Gynkotek, equipped with an ODS Multohyp column (5µ, CS-Chromatographie) and a UV-detector.

<u>HPLC conditions:</u>

Mobile phase: 40 vol% acetonitrile, 60 vol% MilliQ water

Flow rate: 1.1 ml/min

Detection wavelength: 250 nm

Retention times: benzaldehyde 6.9 min, benzoin 9.1 min

The reaction rates were determined by measuring the concentration of the substrate (benzaldehyde) and the product (benzoin) over time using calibration curves (**Appendix**, **Fig. A1, A2**). For progress curve analysis, the concentrations of both substrate and product were measured; whereas only the accumulation of product was used for calculation of initial reaction rates.

One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 µmol benzoin per minute under standard conditions.

II.4.4.5. Determination of benzoin synthesis in supercritical CO_2

Eppendorf tubes (1.5 ml) containing the enzymes BAL or BFD*H281A* (each 200 µg, with cofactor concentration 100-fold higher than in the standard enzyme preparation described in **II.4.1**) were placed in an autoclave (30 ml volume). The substrate benzaldehyde (30 µl) was added on the bottom of the autoclave. The autoclave was heated to 40°C and afterwards the CO_2 (100 bar) was introduced. The reaction was allowed to procede for 48 hours; afterwards, the autoclave was put on ice and slowly depressurized. Thereafter, the autoclave was washed with 3 ml acetonitile to collect the produced benzoin and the remaining benzaldehyde. Their concentrations were determined using HPLC procedure described in **II.4.4.4.**

II.4.5. Determination of enzyme stability

II.4.5.1. Determination of thermostability

To determine the thermostability the enzyme samples were incubated in a water bath at 4, 20, 30, and 40°C. Samples were taken at appropriate time intervals and assayed for residual activity at 30°C using the benzoin cleavage assay for BAL (**II.4.4.1**) and the benzoylformate decarboxylase assay for BFD*H281A* (**II.4.4.2**), when cosolvents were absent[2]. The thermostability of enzyme samples containing cosolvents were analysed using the HPLC benzoin formation assay (**II.4.4.4**). The enzyme deactivation was determined by fitting the experimental data to the exponential decay law:

[2] HL-ADH does not tolerate organic solvents.

$$V_1 = V_0 \cdot e^{-k_{deact} \cdot (t_1 - t_0)}$$ (**eq. 2**),

where V_1 is the activity at the end of the measurement, V_0 - the activity at the beginning of the measurement, k_{deact} – deactivation rate, t_1 and t_0 – time at the end and time at the start of measurement, respectively.

Half-life is defined as the time at which the activity is halved and is calculated according to the following equation:

$$t_{1/2} = \frac{\ln(2)}{k_{deact}}$$ (**eq. 3**)

II.4.5.2. Determination of enzyme stability in non-conventional media

To determine the effect of water-miscible cosolvents on the enzyme stability, the enzyme samples were incubated at 30°C (unless otherwise indicated) with different concentrations of organic solvents or ILs in a water bath. Samples were taken at given time intervals and assayed for activity at 30°C using the HPLC benzoin formation assay (**II.4.4.4**) for both enzymes.

The enzymes and the cofactors are not soluble in ***pure solvents***. In this case, after incubation the samples were centrifuged, the supernatant discarded, and the enzyme redissolved in 50 mM potassium phosphate buffer (pH 8 for BAL, pH 6.5 for BFD*H281A*) containing cofactors (2.5 mM $MgSO_4$ heptahydrate, 0.5 mM ThDP). Thereafter, their residual activity was measured using the HPLC benzoin formation assay.

The enzymes stability under ***supercritical conditions*** was measured at 40°C and 100 bar. The enzyme samples were applied as dry lyophilisates prepared with excess cofactors (100-fold higher than the standard enzyme preparation described in **II.4.1**). After incubation with supercritical CO_2 samples were removed from the autoclave and dissolved in 50 mM potassium phosphate buffer (pH 8 for BAL and 6.5 for BFD*H281A*). The residual activity was measured using the benzoin cleavage assay (**II.4.4.1**) for BAL and benzoylformate decarboxylase assay (**II.4.4.2**) for BFD*H281A*. The minimum incubation time under supercritical conditions was 1 hour because the system needs certain time (~30 min) to reach equilibrium.

II.4.6. Determination of cofactor dissociation

To investigate the cofactor dissociation the enzymes were incubated in 50 mM potassium phosphate buffer/solvent mixtures containing various ThDP concentrations (0.02-0.12 mM) and 2.5 mM $MgSO_4$ heptahydrate. Samples were taken over time and the residual enzyme activity was determined using the 3,3′,5,5′-tetramethoxy-benzoin formation assay (**II.4.3.3**). The residual activity was measured in the same ThDP concentrations as in the incubation buffer.

II.4.7. Determination of enantioselectivity

The enantioselectivity of BAL (20 µg/ml) and BFD*H281A* (100 µg/ml) was tested for two reactions: the benzoin and 2-HPP synthesis in mixtures of organic solvents or ILs with 50 mM potassium phosphate buffer (50 mM, pH 8 for BAL, pH 6.5 for BFD*H281A*), containing 2.5 mM $MgSO_4$, 0.5 mM ThDP. The substrate for benzoin synthesis was 20 mM benzaldehyde, whereas the reaction mixtures for 2-HPP synthesis contained additionally 200 mM acetaldehyde. The reaction temperature was 30°C. Samples were taken after 24 hours to assure complete conversion, extracted with hexane:2-propanol (90:10) and subjected to chiral separation using HPLC instrument from Gynkotek, equipped with a Chiralcel OD-H column from Diacel and a UV-detector. Analysis was performed at room temperature.

HPLC conditions:
Mobile phase: 90 vol% hexane, 10 vol% 2-propanol
Flow rate: 1 ml/min
Detection wavelength: 250 nm
Retention times: benzaldehyde 12.7, 2-*(S)*-HPP 16.1 min, 2-*(R)*-HPP 18.3 min, *(S)*-benzoin 28.5,
(R)-benzoin 40.6 min

The concentrations of products were calculated using calibration curves (**Appendix, Fig. A3**).

II.4.8. Determination of the enzymes spectral properties

II.4.8.1. Tryptophan fluorescence spectroscopy

Tryptophan fluorescence spectroscopy is widely used to determine structural changes in proteins (**Vivian** and **Callis**, 2001). Typically tryptophan residues, which are

exposed to water show maximal fluorescence emission at wavelength of 340-360 nm, when excitated at 280 nm. Triptophan residues, which are totally burried, show a blue-shifted fluorescence emission maximum around 320 nm. Therefore, upon unfolding a red shift can be expected.

Fluorescence spectroscopy was used to detect unfolding of the enzyme molecule. The enzymes (10-20 mg/ml) were incubated in mixtures of organic solvents and ionic liquids with 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP (pH 8 for BAL and pH 6.5 for BFD*H281A*) for 1 h and 24 h at 30°C and subsequently analysed without changing the media. The solvents (except for acetone) did not interfere with the signal. Tryptophan fluorescence spectra were recorded using a Perkin Elmer spectrofluorometer LS 50B. After excitation at 280 nm, the emission was recorded in the range from 320 to 370 nm. Excitation and emission slits were 5 and 7.5 nm, respectively.

II.4.8.2. Circular dichroism spectroscopy

The circular dichroism (CD) spectra of the enzymes (0.5 mg/ml) were obtained by scanning the ellipticity from 300-180 nm using a Jasko J-810 CD spectrophotometer, at medium sensitivity. The nitrogen flow rate was 3 L/min and the quartz cuvette optical pathway was 1 mm. The method was applied to determine the structural melting point and the effect of organic solvents on the enzyme structure.

The melting point is the temperature at which the enzyme loses 50% of its helical structure (measured at 222 nm) and it is defined as the inflection point of the temperature vs. $ellipticity_{222\ nm}$ curve. To determine the melting point, the samples were heated from 25 to 80°C with a heating rate of 1°C/min in 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP (pH 8 for BAL and pH 6.5 for BFD*H281A*) directly in the CD spectrometer prior to recording the CD-spectrum at the same temperatures every 5 minutes. This experiment was carried out in 50 mM potassium phosphate buffer by following the decrease of the helical content at 222 nm upon heating.

To determine the effect of organic solvents on enzyme unfolding, the samples were incubated in the presence of different concentrations of cosolvents in 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP (pH 8 for BAL and pH 6.5

for BFD*H281A*). The CD-spectra were recorded after 5 min (to relate with activity studies) and 24 h incubation (to relate with stability studies).

II.4.9. Determination of pH in cosolvent systems

The pH in water/solvent systems is difficult to measure due to electrostatic interferences. Therefore, a special pH-electrode designed for low-water systems (Solvotrode, Metrohm AG) was used, to allow more consistent measurements. The pH was adjusted using 1 or 10 M HCl and H_3PO_4.

II.4.10. Determination of benzaldehyde and benzoin solubility

Saturated solutions of benzoin and benzaldehyde were prepared by mixing large amounts of the compounds with different concentrations of water-miscible solvents pure water-immiscible solnvents, for 24 h and 30 °C. Thereafter, the mixtures were centrifuged at 13,000 rpm for 1h, the excess amount of the compounds was removed and the rest was centrifuged once again. Samples were taken until constant concentrations were measured and the concentrations of benzaldehyde and benzoin were determined using the HPLC procedure described in **II.4.4.4**.

II.5. Reaction kinetics and mechanism

II.5.1. Macro kinetics by initial rate measurements

The macro kinetic studies were conducted by quantifying the benzoin formation from benzaldehyde within the first 5 minutes of the reaction. The reaction mixture consisted of benzaldehyde (5-60 mM) in potassium phosphate buffer (50 mM), containing 2.5 mM $MgSO_4$, 0.5 mM ThDP and in presence of acetone and DMSO. The reactions were started by addition of 5 µg/ml BAL or 40 µg/ml BFD*H281A*. The benzoin concentration was determined by HPLC using the procedure described in **II.4.4.4**. Initial rates given as kcat [s^{-1}] referred to one monomer of the tetrameric enzymes and were determined by fitting the experimental data into direct v_0-[S] plots.

II.5.2. Micro kinetics by progress curve analysis

To estimate the kinetic parameters, progress curve analysis according to the mechanistic kinetic model was applied. The reactions were followed by monitoring the substrate decrease and the product increase over time using the benzoin formation assay (**II.4.4.4**). The obtained data points for both benzaldehyde and benzoin were used to fit the mechanistic kinetic model (**eq. 4**), which is described in detail by **Zavrel** et al. (2008). Thereafter, the software package gPROMS (version 3.1.3) from Process Systems Enterprises Ltd. (London, UK) was applied. A model simplification is necessary to achieve precise parameter estimated. Thus, the micro-reaction constants k_1 and k_2, as well as k_{-1} and k_{-2} were considered to be identical.

$$v = -\frac{1}{2}\cdot\frac{dA}{dt} = \frac{\frac{k_{catf}}{K_{iA}\cdot K_{mB}}\cdot\left(A^2 - \frac{P}{K_{eq}}\right)}{1+\frac{A}{K_{iA}}\left(1+\frac{K_{mA}}{K_{mB}}\right)+\frac{A^2}{K_{iA}\cdot K_{mB}}+\frac{P}{K_{mP}}+\frac{A\cdot P}{K_{mP}\cdot K_{iB}}} \qquad \textbf{(eq. 4)}$$

The dependent kinetic parameters were calculated according to **eq. 5-7**.

$$K_{iA} = K_{mB} - K_{mA} \qquad \textbf{(eq. 5)}$$

$$K_{mP} = \frac{K_{mB}\cdot\left(K_{mB}-K_{mA}\right)^2\cdot K_{eq}}{2\cdot K_{mA}} \qquad \textbf{(eq. 6)}$$

$$K_{iB} = \frac{K_{mB}\cdot K_{iA}}{K_{mA}\cdot\left[1-\left(\frac{K_{mA}}{K_{iA}}-1\right)\cdot\frac{K_{mP}}{K_{eq}\cdot K_{mB}\cdot K_{iA}}\right]} \qquad \textbf{(eq. 7)}$$

To reduce the number of degrees of freedom the model parameter K_{eq} was determined separately by averaging over all equilibrium data. Based on the estimated values for the independent parameters k_{catf}, K_{mA}, and, K_{mB} the micro-reaction constants were calculated using **eq. 8-11**:

$$k_1 = k_2 = \frac{k_{catf}}{K_{mA}} \qquad \textbf{(eq. 8)}$$

$$k_{-1} = k_{-2} = \frac{k_{catf} \cdot (K_{mB} - K_{mA})}{K_{mA}} \quad \textbf{(eq. 9)}$$

$$k_3 = k_{catf} \quad \textbf{(eq. 10)}$$

$$k_{-3} = \frac{k_{catf}}{K_{eq} \cdot (K_{mB} - K_{mA})^2} \quad \textbf{(eq. 11)}$$

II.5.3. ^{1}H NMR spectroscopy. Qualitative analysis of reaction intermediates

The relative distribution of ThDP and of the acid-stable intermediates HBz-ThDP and benzoin-ThDP can be assessed using a combined acid quench/ ^{1}H NMR- method (**Tittmann** et al., 2003). Thereafter, the net rate constants of the three main reaction steps can be estimated. In this thesis, the distribution of intermediates was determined only qualitatively as a confirmation of the reliability of the mechanistic kinetic model described in **I.3.4** and **II.5.2**).

To remove excess ThDP, the enzymes were repeatedly washed (three times) with 50 mM potassium phosphate buffer at 4°C using a Centricon centrifugal filter unit with molecular cut-off 10 kDa (Millipore). Thereafter, BAL (6 mg/ml) or BFD*H281A* (10 mg/ml) were mixed with 20 mM benzaldehyde in 50 mM potassium phosphate buffer (pH 8 for BAL and pH 6.5 for BFD*H281A*) at 30 °C for 1-2 s to ensure steady-state conditions and stopped by addition of 12.5 (w/v) TCA/1 M HCl (in D_2O).

Subsequently, the precipitated protein was discarded after centrifugation and the supernatant containing the intermediates, substrates and products of the reaction were subjected to 1D ^{1}H NMR spectroscopy at 298 K using water presaturation techniques for suppressing the water signal. The procedure of the sample preparation is depicted on **Fig. 7**.

For assignment and analysis of ThDP, HBz-ThDP and benzoin-ThDP, the 2'-CH_3 and 4-CH_3 ^{1}H NMR singlet signals of ThDP (2.65 and 2.58 ppm), HBz-ThDP (2.47 and 2.42 ppm) and benzoin-ThDP (2.45 and 2.43 ppm) were used.

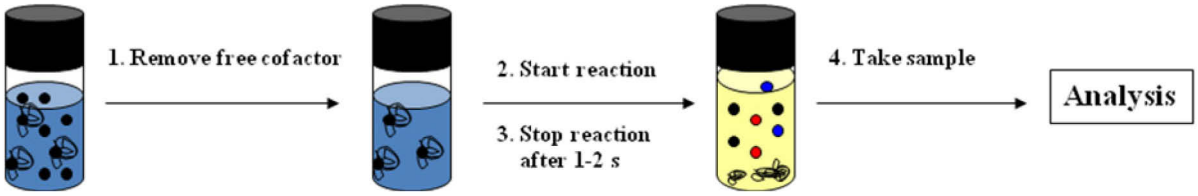


Figure 7: Sample preparation for ^{1}H NMR spectroscopy.

III. RESULTS AND DISCUSSION

III.1. Activity, stability and spectral properties of BAL and BFD*H281A*

III.1.1. Effect of water-miscible organic solvents

In order to determine the optimal reaction conditions for the enzymatic synthesis of benzoin, different concentrations of water-miscible organic solvents (DMSO, acetone, ethanol and 2-propanol) were tested with BAL and BFD*H281A*. The soluble protein content in all cases was measured, since precipitation of protein in solvents could be expected. Additionally, pH effects have been studies, as some media caused changes of the apparent pH (**Appendix, Fig. A4**).

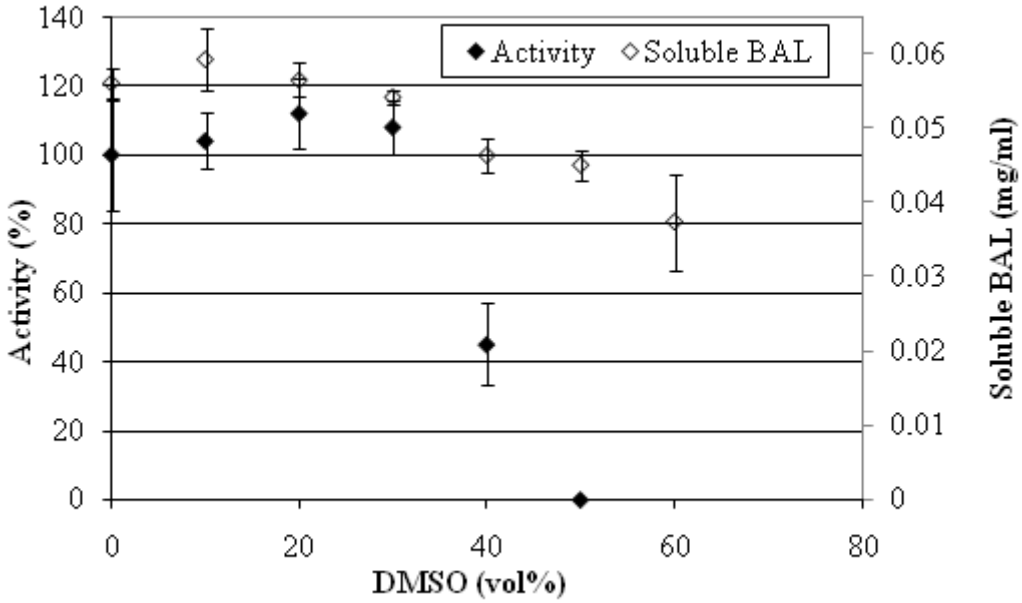
Stability studies were performed in DMSO (30 vol% with BAL and 10-20 vol% with BFD*H281A*) at different temperatures. The activity of both enzymes was measured at different pH (BAL activity - at pH 8 and BFD*H281A* activity – at pH 6.5) because they have different pH requirements. The circular dichroism and fluorescence spectra of both enzymes in increasing concentrations of cosolvents were measured and analyzed to elucidate conformational changes that may occur under these non-conventional conditions.

III.1.1.1. DMSO as a cosolvent. Effect of pH and temperature

DMSO is the most frequently used cosolvent for reactions involving BAL and BFD, since it was firstly suggested by Prof. Demir to improve the substrate solubility (**Demir** et al., 1999). Interestingly, 20-30 vol% DMSO improved not only the substrate solubility but also BAL's activity (**Janzen** et al., 2006) and stability (**Stillger**, 2004). A positive effect of DMSO was observed also with several BFD variants (**Lingen** et al., 2002). In this thesis, the effect of DMSO on the enzyme activity and stability was studied in more detail, in order to determine the most important parameters which are involved in the activation and inactivation mechanism of the enzymes in presence of water-miscible solvents.

III.1.1.1a. Enzyme activity in DMSO/aqueous buffer mixtures

In the presence of increasing concentrations of DMSO, the initial reaction rates of BAL without adjusting the pH demonstrated a small peak of activity at 20-30 vol % DMSO (**Fig. 8**). An explanation for this phenomenon could be that the addition of DMSO to potassium phosphate buffer increased the apparent pH, which resulted in increased enzyme activity.



DMSO (vol%)	pH (-)
0	8.00
10	8.24
20	8.46
30	8.68
40	8.88
50	9.08

Figure 8: Initial reaction rates and soluble protein content of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and DMSO, with 20 mM benzaldehyde. Mean values of three independent experiments are shown. Apparent pH-shift by DMSO is shown in the table.

Thus, the deactivation effect of the solvent competed with the activation effect of increased pH, as the initial pH (8) was lower than the optimum for the enzyme in potassium phosphate buffer (**Fig. 9**).

When the experiment was repeated at adjusted pH (pH 8 for all DMSO concentrations), the enzyme was deactivated gradually until 40 vol% DMSO, where no activity was observed any more (**Fig. 10).** This experiment demonstrates that the apparent pH-shift in the presence of DMSO, which is measured with a pH glass electrode, affects BAL activity, suggesting that the pH-shift is not an artifact caused by the electrode.

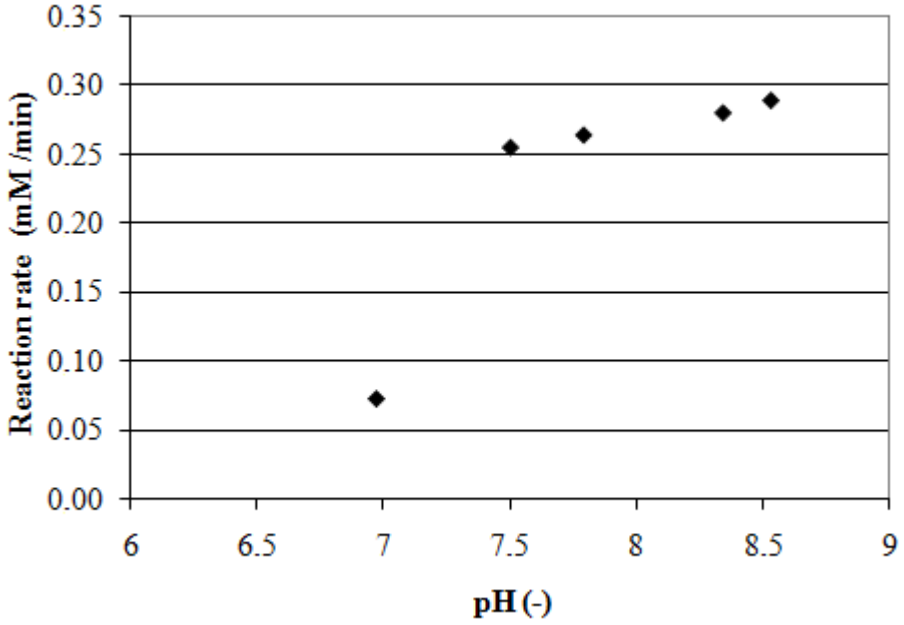


Figure 9: pH-optimum oft he BAL-catalysed benzoin formation in 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) with 20 mM benzaldehyde. Activity at pH > 8.5 was not measured due to cofactor precipitation.

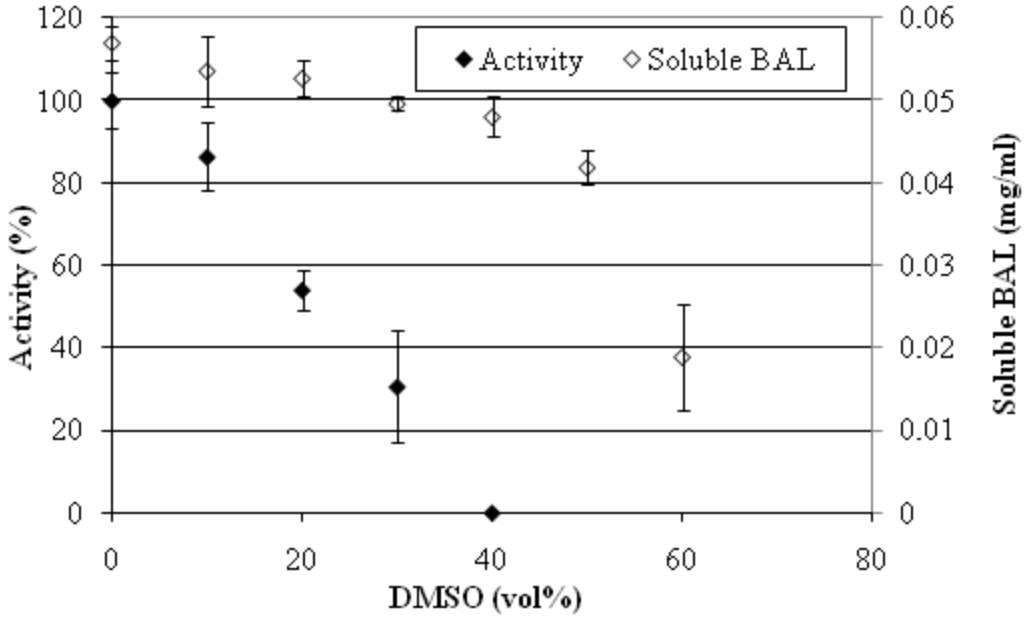
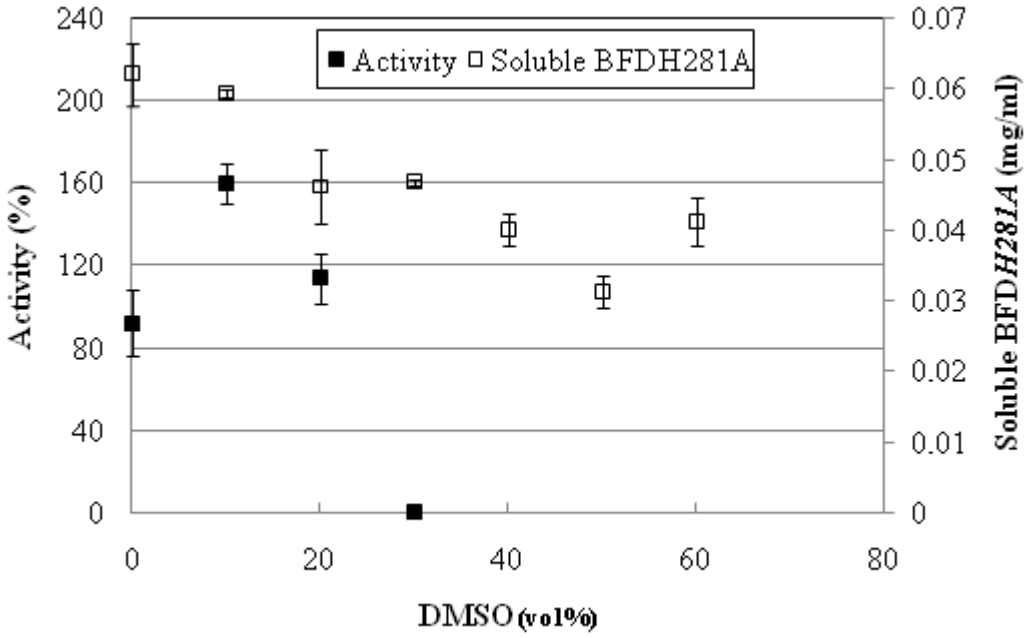


Figure 10: Initial reaction rates and soluble protein content of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and DMSO, with 20 mM benzaldehyde. pH adjusted to 8. Mean values of three independent experiments are shown.

For BFD*H281A* (**Fig. 11**), the addition of 10 vol% DMSO caused 60% increase of the reaction rate, whereas further addition of DMSO reduced the initial rate activity.

Interestingly, when the pH was kept constant (pH 6.5), no significant difference of respective reaction rates were observed (**Fig. 12**).



DMSO (vol%)	pH (-)
0	6.52
10	6.70
20	6.92
30	7.37

Figure 11: Initial reaction rates and soluble protein content of BFD*H281A*-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and DMSO, with 20 mM benzaldehyde. Mean values of three independent experiments are shown. Apparent pH-shift by DMSO is shown in the table**.**

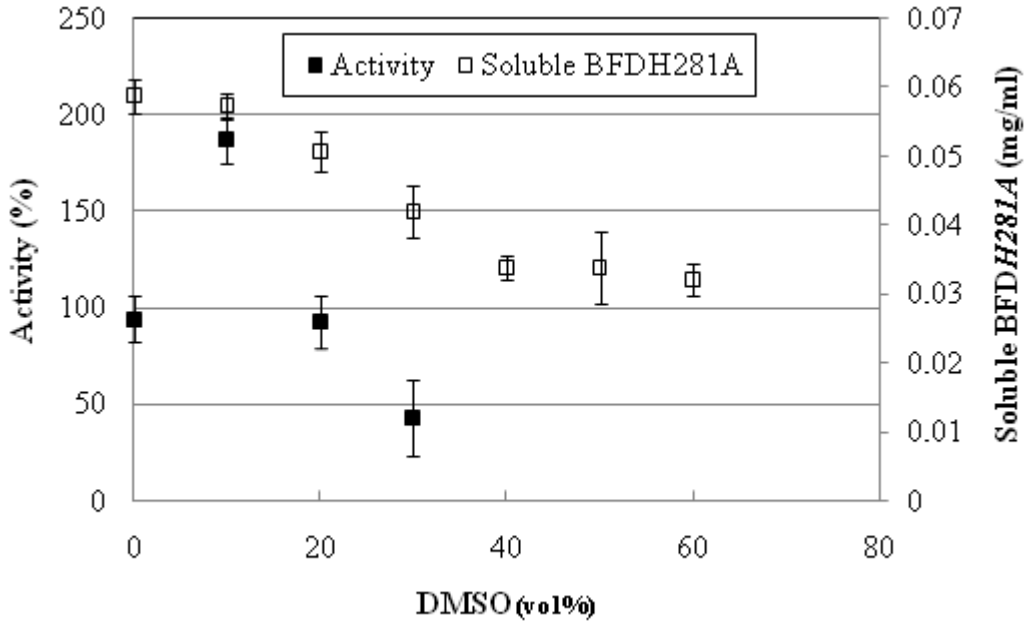


Figure 12: Initial reaction rates and soluble protein content of BFD*H281A*-catalysed benzoin formation in mixtures 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and DMSO, with 20 mM benzaldehyde. pH adjusted to 6.5. Mean values of three independent experiments are shown.

These unexpected results showed that the effect of the pH-shift is more complex and not the only explanation for the observed phenomenon. Therefore, additional experiments to determine the pH-optimum of the benzoin formation for each respective concentration of DMSO (0, 10, 20, and 30 vol%) were conducted. **Fig. 13** shows that with increasing concentrations of DMSO the pH-optimum apparently shifts to the alkaline range.

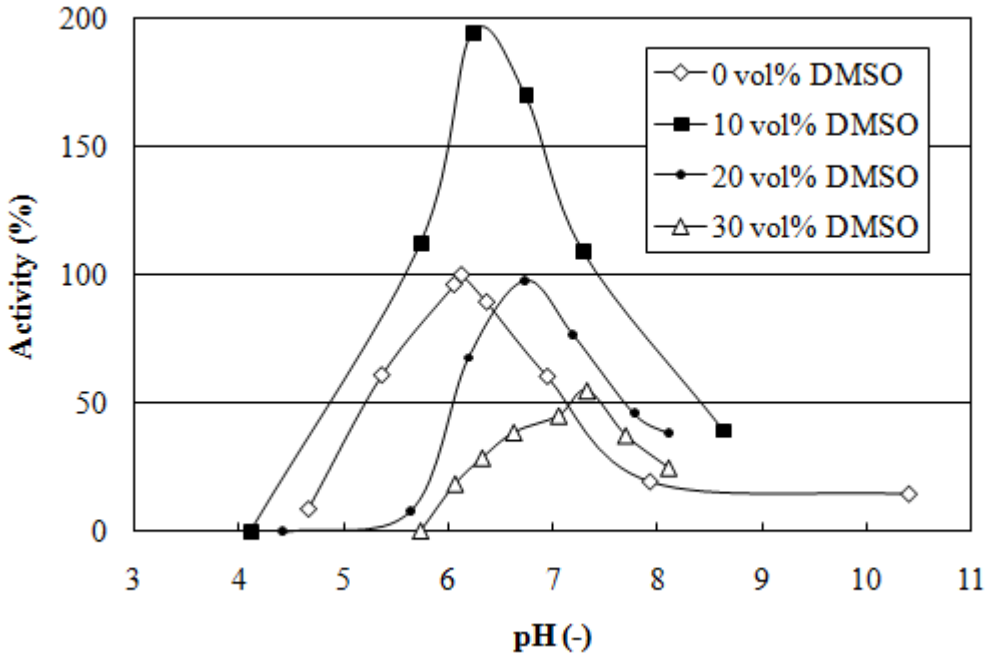


Figure 13: pH-optimum of BFD*H281A*-catalyzed benzoin formation. 100% activity is determined as the activity in 0 vol% DMSO. Optimum pH as follows: 0 vol% DMSO: pH 6.13; 10 vol% DMSO: pH 6.23; 20% DMSO: pH 6.73, 30 vol% DMSO: pH 7.33.

It is known that the pH-range of enzyme activity is determined mainly by the pKa values of the active site residues (**Nielsen** and **McCammon**, 2003) and in this case also by the pKa of the cofactor ThDP, especially the pKa values of the two unstable zwitterionic intermediates: the C2- carbanion and the C2α-carbanion (enamine) (**Jordan** et al. 2000; **Nemeria** et al, 2007). However, the pKa values are solvent-dependent. Therefore, a change in the solvent composition could affect the pH requirement of the enzyme. This experiment clearly shows that the addition of cosolvents to aqueous buffer results in a completely new solvent system. Adjusting the pH (only as an apparent value read by the pH-electrode) does not provide equal reaction conditions due to other factors which are not kept constant: water activity, solvent polarity, ionic strength, etc.

Another factor that affects the reaction rates in different concentrations of organic solvents is the ***temperature***. Without cosolvent, activity of BAL was improved by only 20%, when the reaction temperature was increased from 30°C to 40°C. As these are initial rate measurements, which were conducted in the range of 5-10 min this minor increase of activity is partly due to the low stability of BAL at 40°C. Intriguingly, the combination of elevated temperature and addition of DMSO resulted in acitivity improvement of 280 % – a valuable example of the advantage of using cosolvent systems (**Fig. 14**).

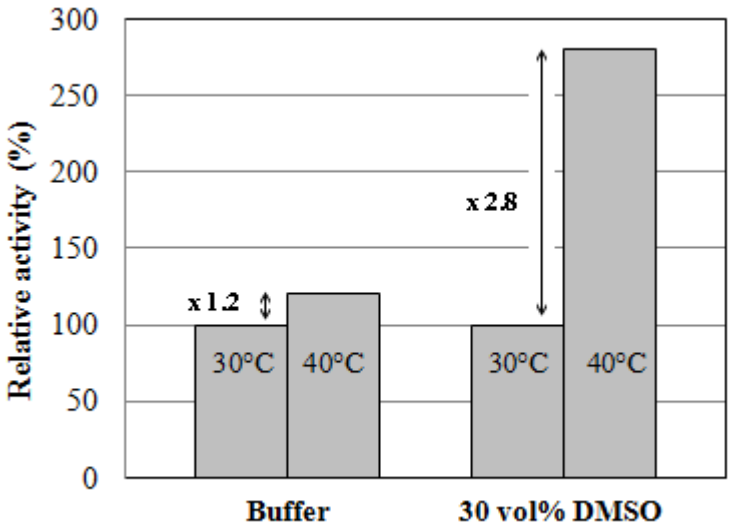


Figure 14: Activity of BAL in 50 mM potassium phosphate buffer (2.5 mM MgSO4, 0.5 mM ThDP, pH 8) with and without 30 vol% DMSO at 30°C and 40°C.

III.1.1.1b. Enzyme stability in DMSO/aqueous buffer mixtures

The influence of 30 vol% DMSO on the stability of BAL was investigated at 4°C, 30°C and 40°C, and was compared to the stability in 50 mM potassium phosphate buffer at same temperature. At 4°C (storage conditions), the half-life in buffer and in 30% DMSO was identical (8-10 d, **Fig. 15**), whereas at 30°C the stabilizing effect of DMSO was noticeable, with half-life of BAL increased by a factor of 3 (**Fig. 16**).

When the experiments were carried out at 30°C over several days, one reason for the more rapid deactivation of BAL in phosphate buffer could be bacterial growth in the phosphate buffer, which might be suppressed by 30 vol% DMSO. To further investigate this aspect the experiment was repeated under sterile conditions. However, the data presented in **Fig. 17** clearly demonstrate that bacterial growth can be ruled out as a reason for this phenomenon as there are no significant differences in enzyme stability compared to

non-sterile conditions (**Fig. 16**). In both cases addition of 30 vol% DMSO caused similar stabilization.

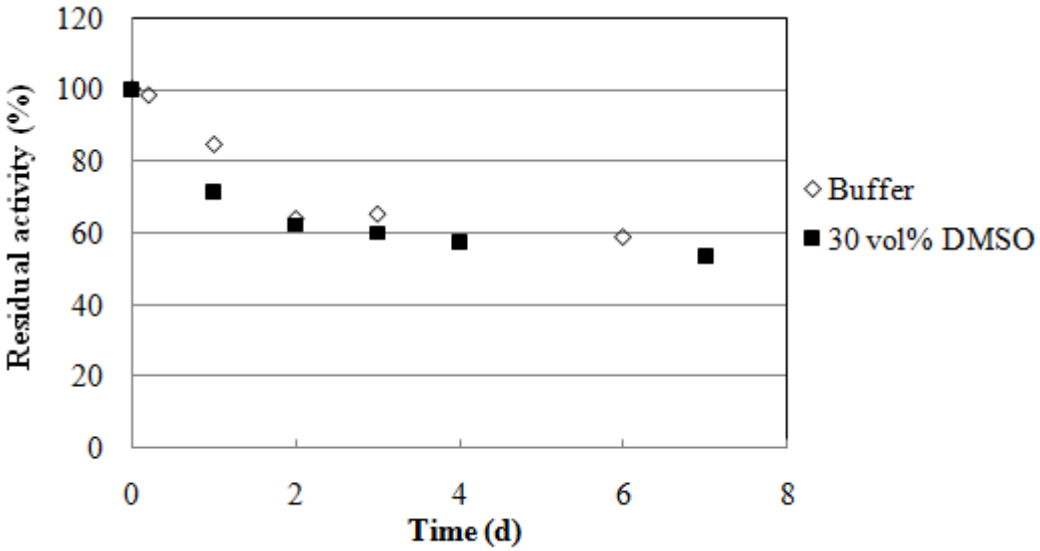


Figure 15: Stability of BAL in 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) with and without 30 vol% DMSO, 4°C, pH adjusted to 8.

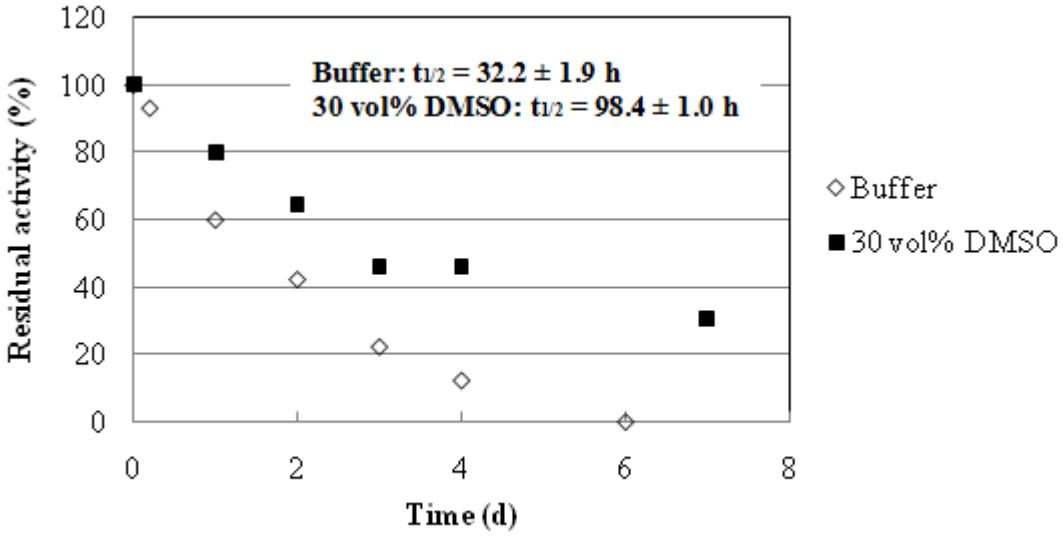


Figure 16: Stability of BAL in 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) with and without 30 vol% DMSO, 30°C, pH adjusted to 8.

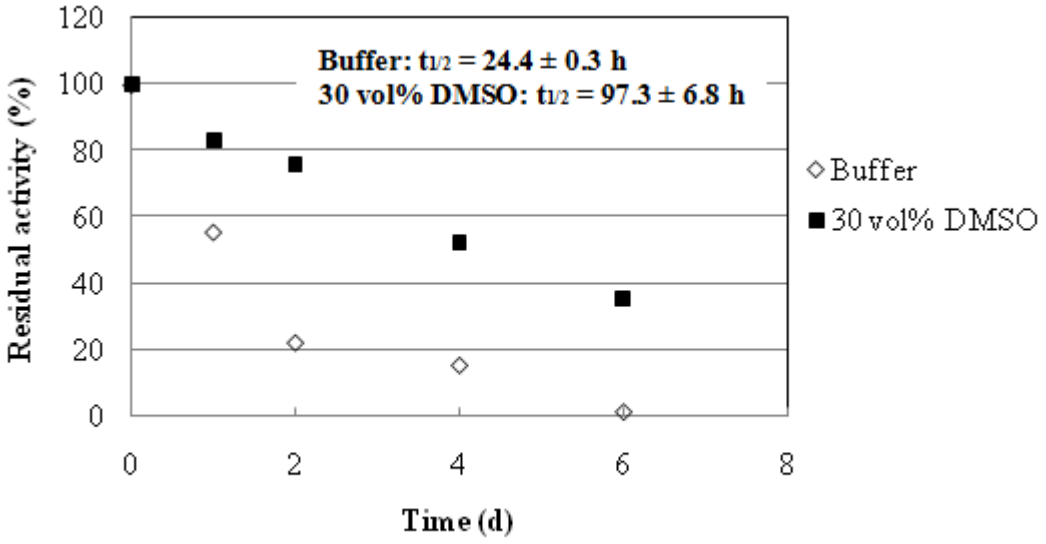


Figure 17: Stability of BAL in 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) with and without addition of 30 vol% DMSO, 30°C, pH adjusted to 8 (sterile conditions).

Compared to BAL the stability of BFD*H281A* was much higher. In phosphate buffer, pH 6.5, no decrease of activity was observed at 30°C within 200 hours (**Fig. 18**). In contrast to BAL, addition of 10-20 vol% DMSO caused a slight decrease of the stability.

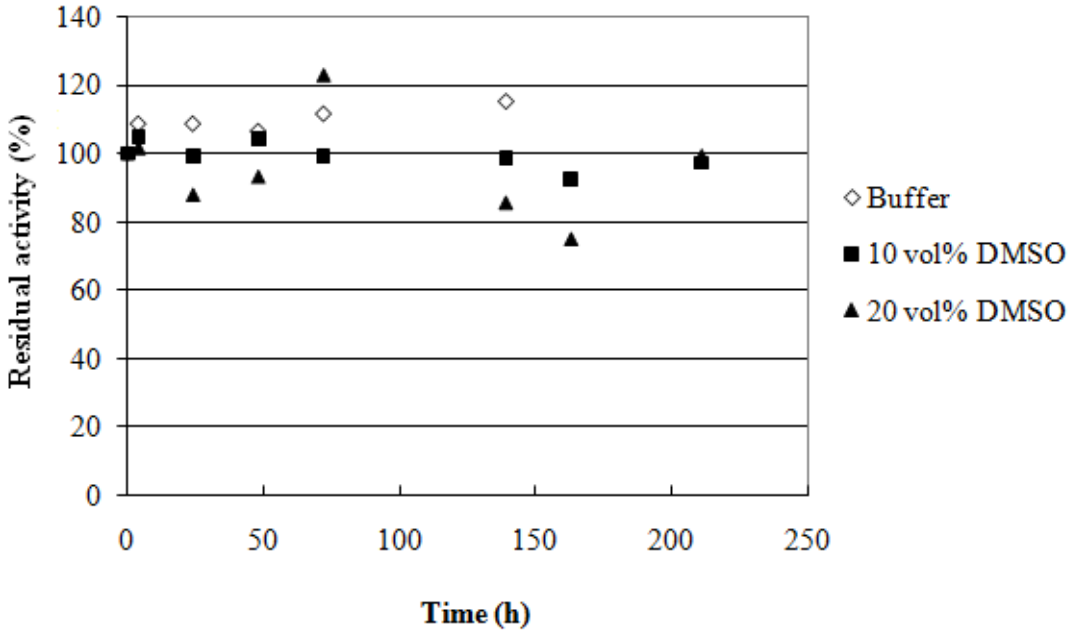


Figure 18: Stability of BFD*H281A* in 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) with and without DMSO (10-20 vol%), 30°C, pH adjusted to 6.5.

At 40°C half-lives of both enzymes decrease and the half-life of BAL was only 1.3 h in phosphate buffer and 4.5 h in 30 vol% DMSO, respectively (**Fig. 19**). Not surprisingly, BFD*H281A* showed much higher stability, with a half-life around 50-60 h in buffer, 10 and 20 vol% DMSO (**Fig. 20**). An interesting feature of BFD*H281A* is that its activity always increased within the first hours of incubation.

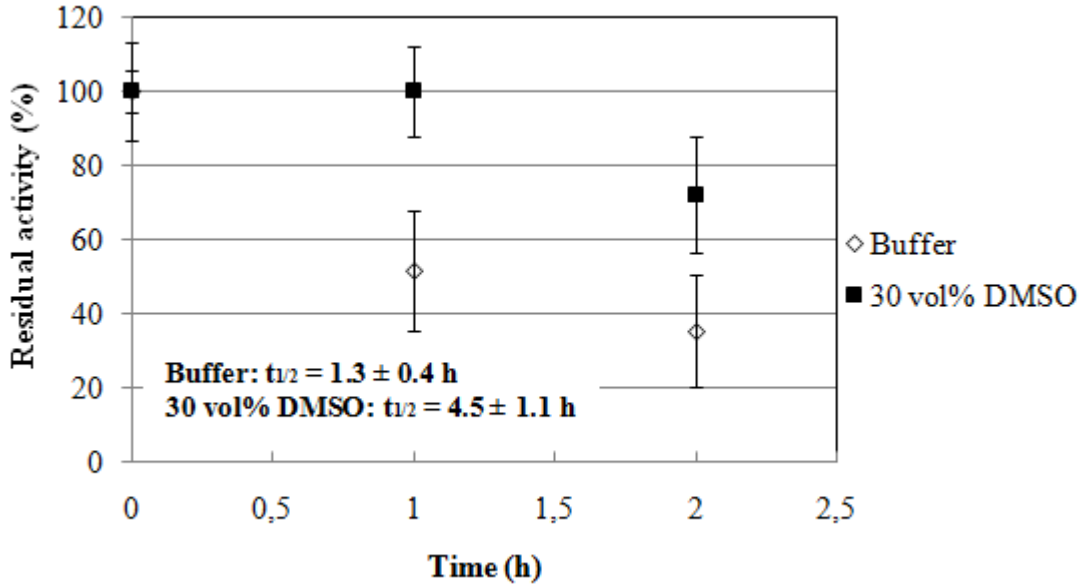


Figure 19: Stability of BAL in 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) with and without 30 vol% DMSO, 40°C. pH adjusted to 8. Mean values of three separate experiments are shown.

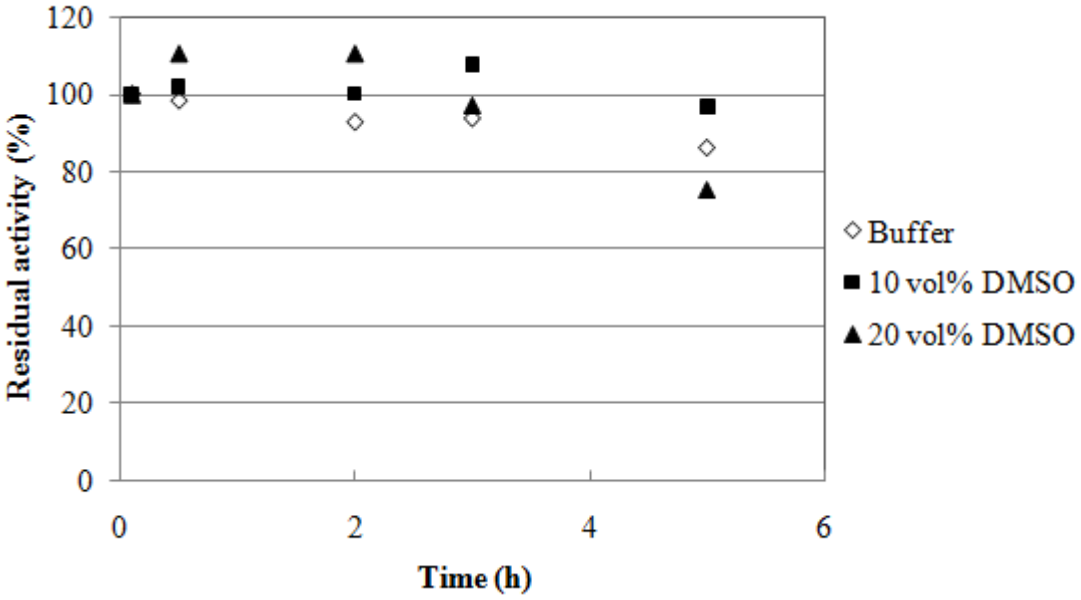


Figure 20: Stability of BFD*H281A* in 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP, pH 6.5) with and without DMSO (10-20 vol%). pH adjusted to 6.5, 40°C. Experiment performed once.

The main reason to perform this experiment at 40°C was to find out whether the enzymes could be stable also in $scCO_2$. 35°C is the supercritical temperature for CO_2; therefore a temperature of 40°C is usually maintained in the autoclave in order to ensure supercritical conditions. In this respect $scCO_2$ could be an appropriate solvent for BFD*H281A*, but not for BAL.

III.1.1.2. Acetone, ethanol and 2-propanol as cosolvents

The initial reaction rates of both enzymes were tested in mixtures of aqueous buffer and further organic solvents. Solvents of different functionality were chosen to resemble DMSO in their ability to improve the benzaldehyde and benzoin solubility (**Appendix**, **Table A4**) and to possess 100% miscibility with water. Examples show that addition of acetone in aqueous media (**Kermasha** et al. 2001) and treatment of enzymes with acetone prior to lyophilisation (**Wu** et al. 2007) can considerably improve the enzyme activity. The acetone molecule and DMSO molecule have similar structures with only one difference in the central atom; it is carbon for acetone and sulfur for DMSO. Furthermore, acetone molecule is planar, whereas DMSO molecule is tetraedric with three valences occupied by two methyl groups and an oxygene atom, and one free electrone pair. Therefore, it is interesting to investigate the differences of their effect on enzyme properties. Ethanol is of particular interest because it has already been successfully used with BFD variants (**Lingen** et al., 2002), as it could slightly improve the enzyme activity in ethanol/aqueous buffer mixtures. 2-propanol contains the same functional group as ethanol (alchohol) but in addition could be expected to be a better solvent because of its higher log P value (see **Introduction**, **I.2.2.1** and **Appendix**, **Table A5**).

Furthermore, acetone, ethanol and 2-propanol are sometimes used in protein purification procedures to precipitate native proteins (**Scopes**, 1993), which shows that enzymes do not get easily inactivated when treated with these solvents.

Figures 21-23 depict the dependence of the enzymes activities and concentration on the concentrations of respective organic solvent. Although BFD*H281A* shows a slightly lower deactivation rate in the presence of solvents than BAL, in all cases both enzymes were completely and irreversibly inactivated in the presence of 30 vol% cosolvent.

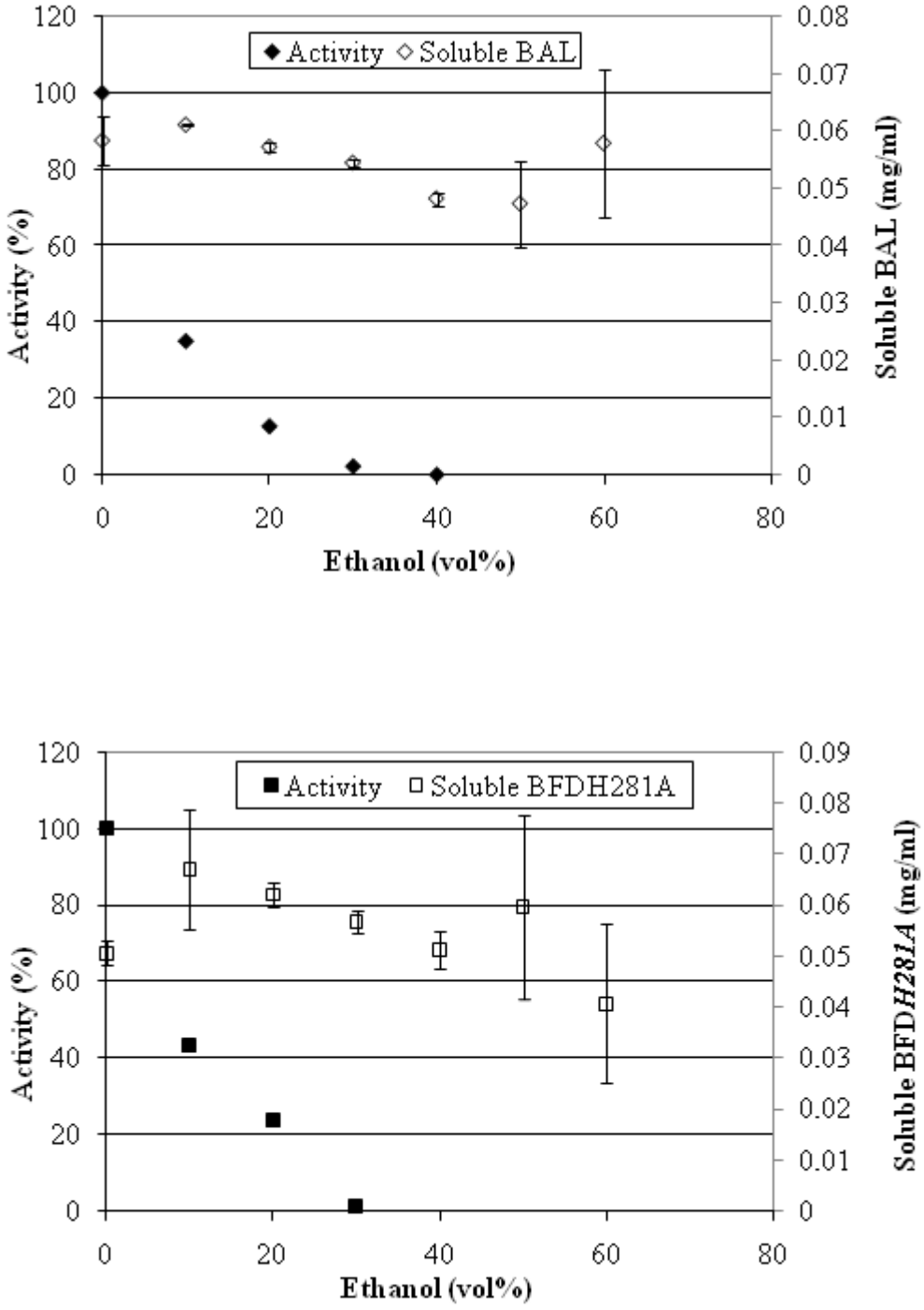
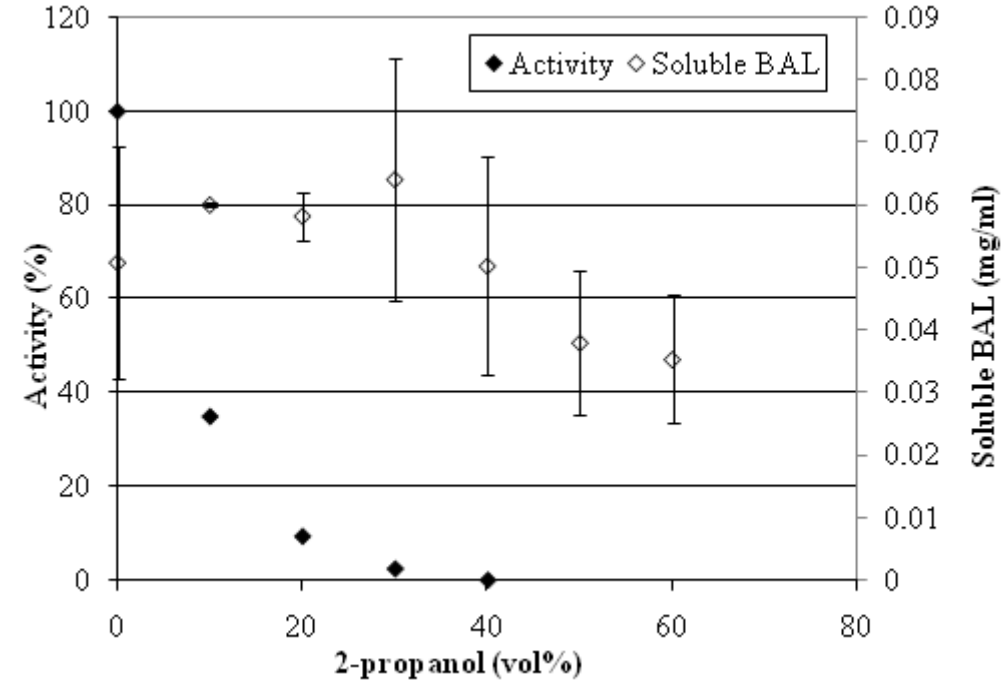


Figure 21: Initial reaction rates of the BAL- and BFD*H281A*-catalyzed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 8: BAL, pH 6.5: BFD*H281A*) and ethanol, with 20 mM benzaldehyde. pH not adjusted. Activity measurements performed once, protein content is a mean value of three measurements.



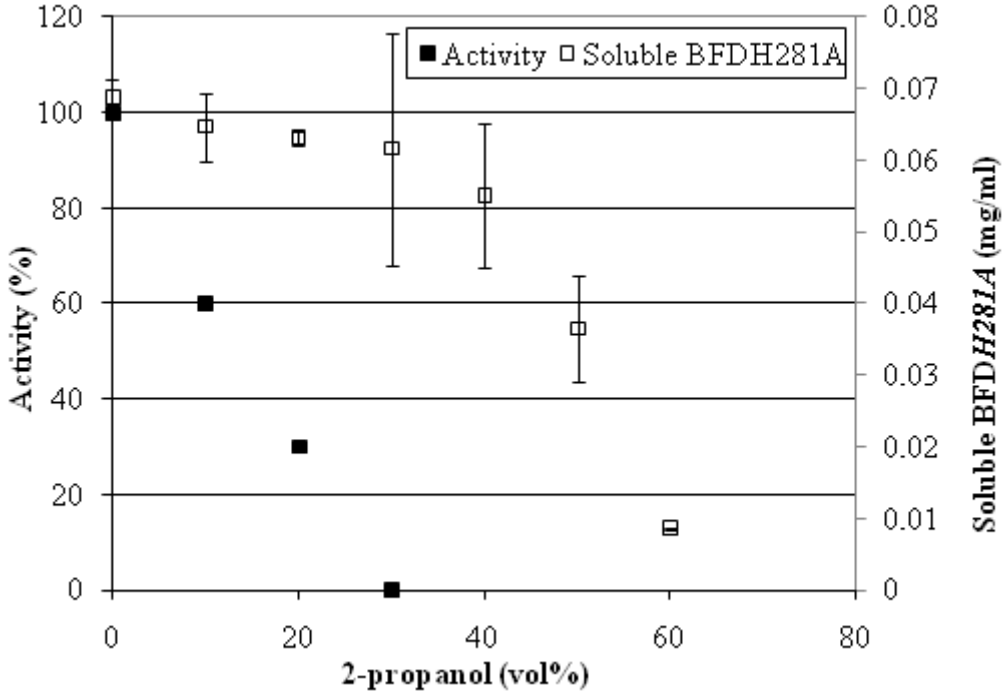


Figure 22: Initial reaction rates of the BAL- and BFD*H281A*-catalyzed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 8: BAL, pH 6.5: BFD*H281A*) and 2-propanol, with 20 mM benzaldehyde. pH not adjusted. Activity measurements performed once, protein content is a mean value of three measurements**.**

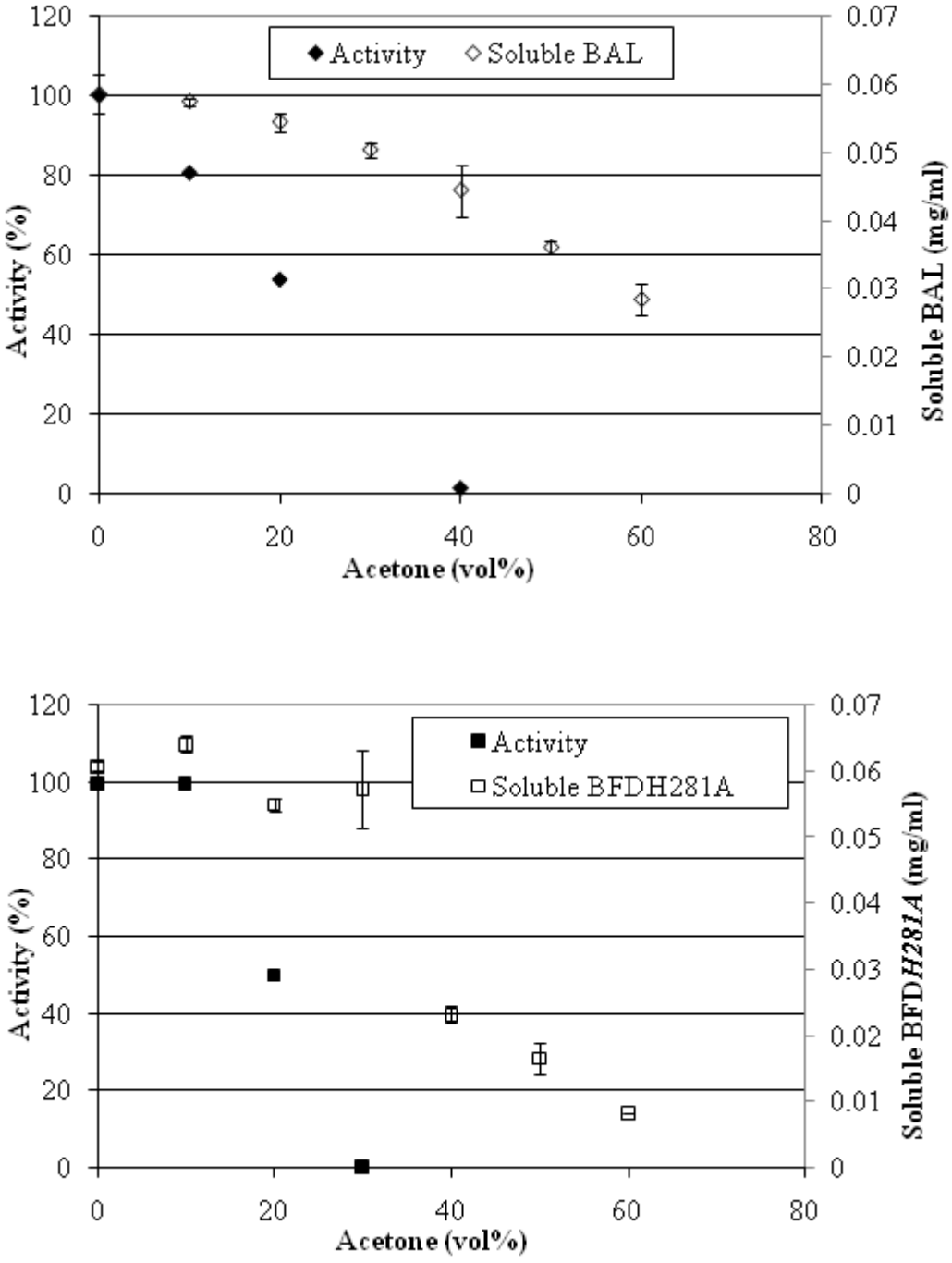


Figure 23: Initial reaction rates of the BAL- and BFD*H281A*-catalyzed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 8: BAL, pH 6.5: BFD*H281A*) and acetone, with 20 mM benzaldehyde. pH not adjusted. Activity measurements performed once, protein content is a mean value of three measurements.

Under all conditions tested with acetone, ethanol and 2-propanol both enzymes showed similar deactivation trends. However, the effect of these cosolvents was very different compared to the effects observed with DMSO despite the similar alkaline pH-shift, which was observed in all solvents (**Appendix**, **Fig. A4**).

Within the range of 0-30 vol% cosolvent deactivation can partially be due to enzyme precipitation, which was most pronounced in presence of acetone (**Fig. 23**).

Attempts were made to correlate various solvents properties (**Appendix**, **Table A5**) with the enzyme inactivation they cause. According to the denaturation capacity criterion (**Khmelnitsky** et al. 1991) ethanol should be better solvent than acetone because its denaturation capacity is lower. The log P criterion (**Introduction**, **I.2.2.1**) also does not explain the phenomenon, as it is mainly applicable for non-aqueous media. The effect of the solvent nature is obviously very complex and it largely depends on the enzyme structure.

The water activity decreases in increasing concentrations of organic cosolvents (**Bell** et al., 1997); therefore, this effect alone can explain the slightly lower deactivation rate of BFD*H281A* in increasing cosolvent concentration (i.e. decreasing water activity) as this enzyme is known to prefer lower water activity (50%) at least under gas phase conditions (**Mikolajek** et al, 2007). However, the effect of water activity is complex and can vary depending on the conditions used. For instance, the best activity of BAL in a gas phase was found to be in 100% water activity (**Mikolajek** et al. 2007) but the enzyme remains more active in DMSO than in ethanol, although the water activity in increasing DMSO concentrations drops faster (**Appendix**, **Fig. A6**; **Bell** et al., 1997), which is an indication that the enzyme inactivation under gas phase conditions and in solution follows a different pattern.

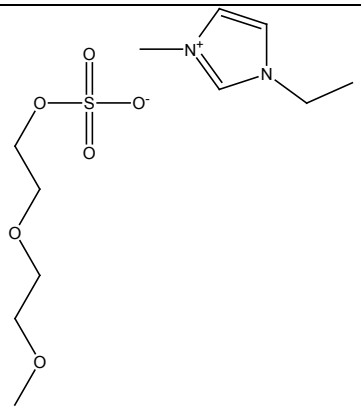
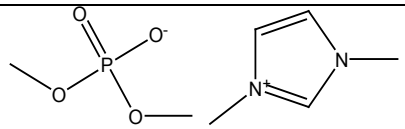
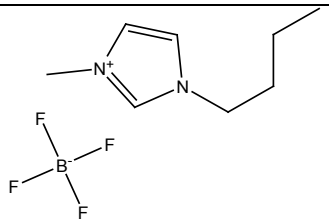
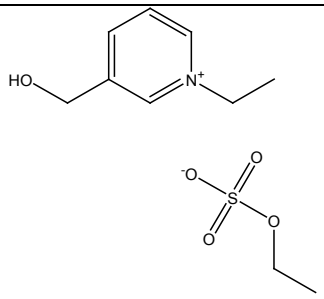
The stability of both enzymes was tested only in pure water-miscible organic solvents. The results are presented and discussed in **III.2.1**.

III.1.2. Effect of water-miscible ionic liquids

The initial reaction rates of both enzymes were further measured in the presence of increasing concentrations of water-miscible ionic liquids. The tested water-miscible imidazolium- and pyridinium- cation, as well as sulftate-, phosphate-, and tetrafluoroborate-anion containing ionic liquids are shown in **Table 3**. The selected ILs contained stabilizing and destabilizing anions to investigate their effect on the enzyme properties and to find out whether the results wll be in agreement with previously observed

trends of the behavoir of ions in Hofemster series (**Introduction**, **I.2.1.2**, **Zhao** et al. 2006[a,b,c].

Table 3: Ionic liquids used in this thesis.

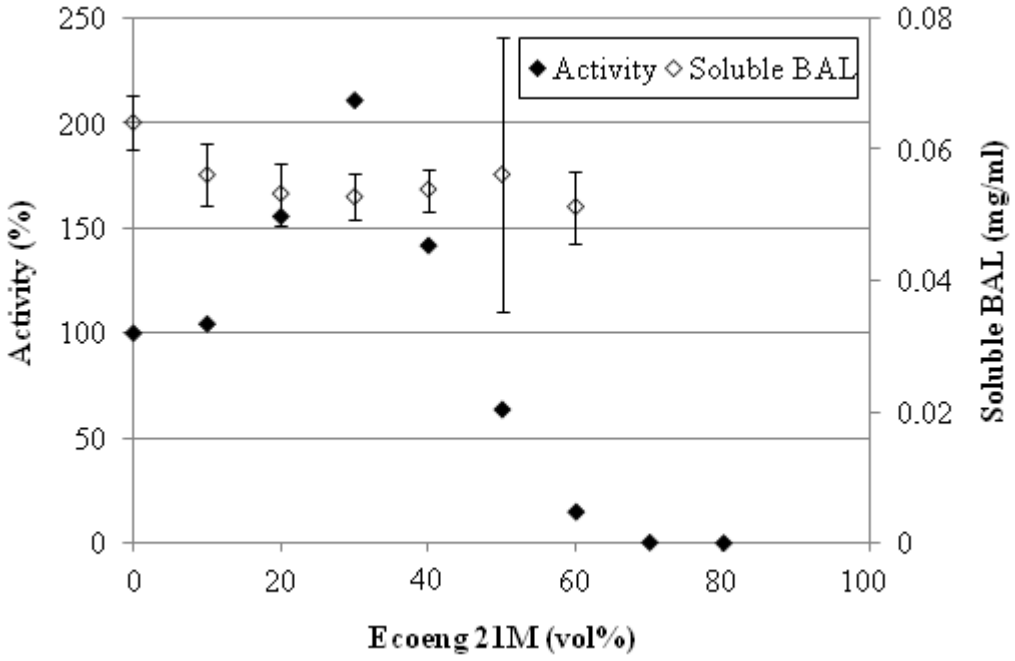
Ionic liquid	Structural formula
Ecoeng 21M **(1-ethyl-3-methyl imidazolium diethyleneglycolmonomethylether sulfate)** **MW: 310.3672 g/mol**	
Ecoeng 1111P **(1,3-dimethyl-imidazolium dimethylphosphate)** **MW: 222.1788 g/mol**	
BMIM.BF_4 **(1-butyl-3-methylimidazolium tetrafluoroborate)** **MW: 226.0227 g/mol**	
EHMPES **(1-ethyl-3-hydroxymethylpyridinium ethylsulfate)** **MW: 263.0827 g/mol**	

III.1.2.1. Ecoeng 21M as a cosolvent

III.1.2.1a. Enzyme activity in Ecoeng 21M/aqueous buffer mixtures

Ecoeng 21M had very different effects on the activity of BAL and BFD*H281A*. Addition of increasing concentrations of the compound led to significant increase of BAL

activity, peaking at 30 vol% with a more than two-fold higher activity compared to aqueous buffer. However, higher concentrations led to fast deactivation and in 70 vol% Ecoeng 21M BAL was no longer active (**Fig. 24**).



Ecoeng 21M (vol%)	pH (-)
0	8.02
10	8.09
20	8.17
30	8.26
40	8.34
50	8.40
60	8.49
70	8.54

Figure 24: Initial reaction rates of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 8) and Ecoeng 21M, with 20 mM benzaldehyde. pH not adjusted. Activity measurements performed once, protein content is a mean value of three measurements.

When the pH was kept constant at pH 8 (**Fig. 25**), the overall activity was decreased. A peak of 1.5-fold improved activity was observed in 20 vol% Ecoeng 21M, whereas in 60 vol% Ecoeng 21M the enzyme was completely deactivated. In both cases (**Fig. 24, 25**), the amount of soluble enzyme did not decrease significantly upon addition of the ionic liquid.

When investigating the enzyme properties in water/cosolvent mixtures it is important to note the difference in the pH-shift occuring in organic solvents and ionic liquids. The pH-increase in presence of organic solvents is due to dilution of H^+ present in the solution; therefore, the pH of the aqueous buffer in the presence of organic solvents changes similarly (**Appendix**, **Fig. A4**). ILs are more complicated in that respect because of their various acidic and basic groups (**Appendix**, **Fig. A5**); therefore addition of ILs to aqueous buffer can cause an increase or decrease of pH.

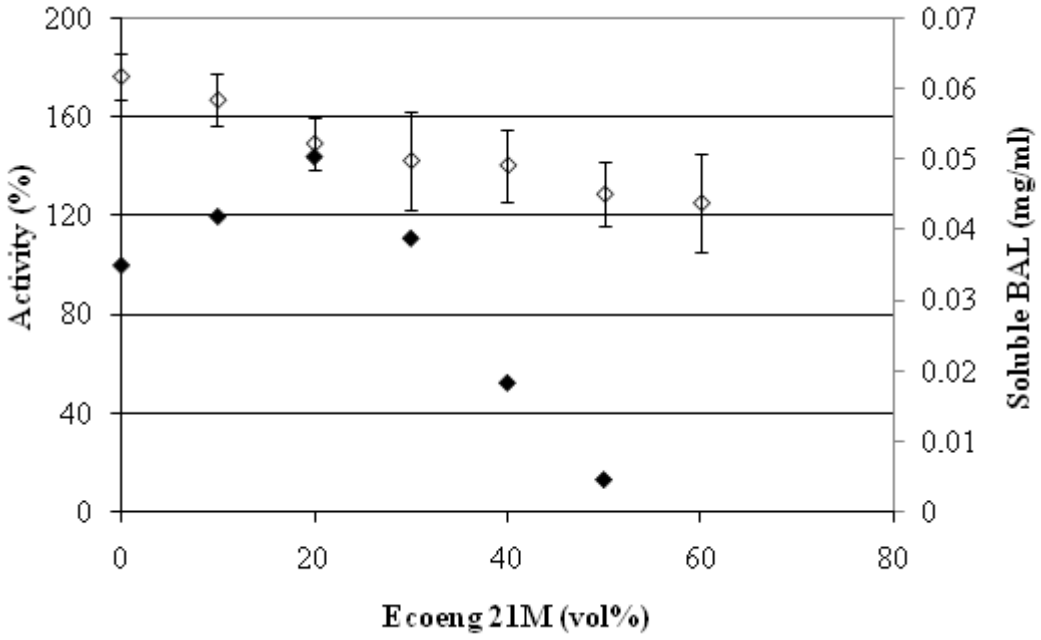
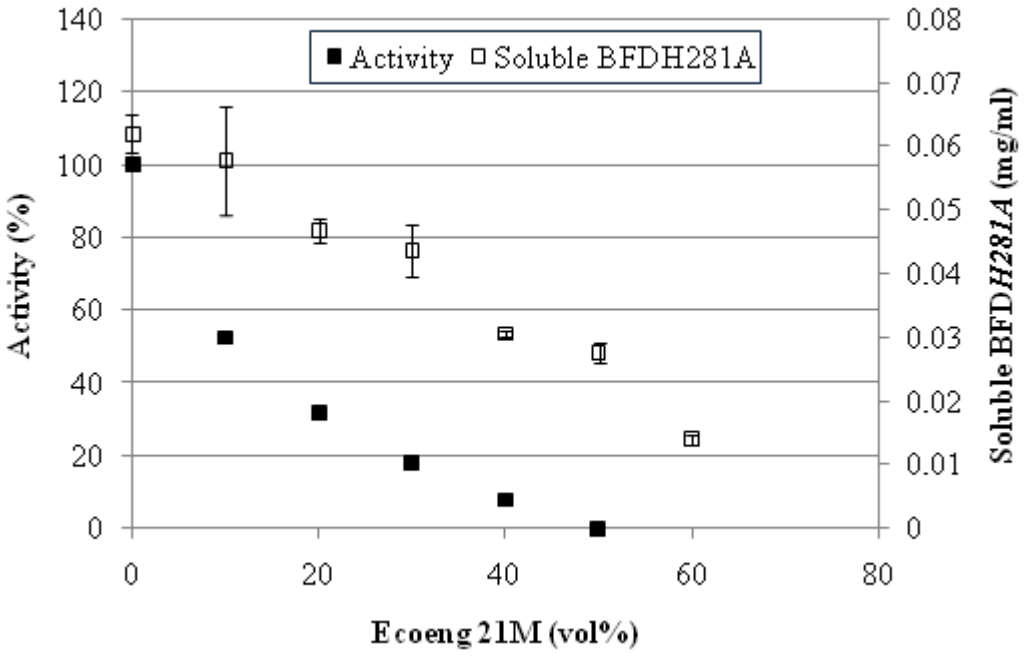


Figure 25: Initial reaction rates of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 8) and Ecoeng 21M, with 20 mM benzaldehyde. pH adjusted to 8. Activity measurements performed once, protein content is a mean value of three measurements.

In contrast to BAL, BFD*H281A* shows highest activity in aqueous buffer. Any addition of Ecoeng 21M led to deactivation of the enzyme and the enzyme was completely inactive in 50 vol% Ecoeng 21M (**Fig. 26**). The loss of activity upon addition of Ecoeng 21M observed with BFD*H281A* is accompanied by significant loss of soluble protein content, which is comparable with the effect of acetone on both enzymes (**Fig. 23**).

As mentioned in **III.1.1**, both enzymes have different pH requirement for activity; therefore it is not always possible to investigate the enzymes under the same conditions. To find out whether the precipitation of BFDH*281A* was due to the lower pH and to compare both enzymes on the same basis, the solubility of BAL in different concentrations of Ecoeng 21M at pH 6.5 was measured. Under these conditions, BAL solubility was similar to that of BFD*H281A* **(Fig. 27)**, i.e. both enzymes precipitate at pH 6.5 in presence of Ecoeng 21M. As already shown (**Fig. 9**), BAL showed no activity at this pH.



Ecoeng 21M (vol%)	pH (-)
0	6.50
10	6.60
20	6.74
30	6.81
40	6.86
50	6.92

Figure 26: Initial reaction rates of BFD*H281A*-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 6.5) and Ecoeng 21M, with 20 mM benzaldehyde. pH not adjusted. Activity measurements performed once, protein content is a mean value of three measurements.

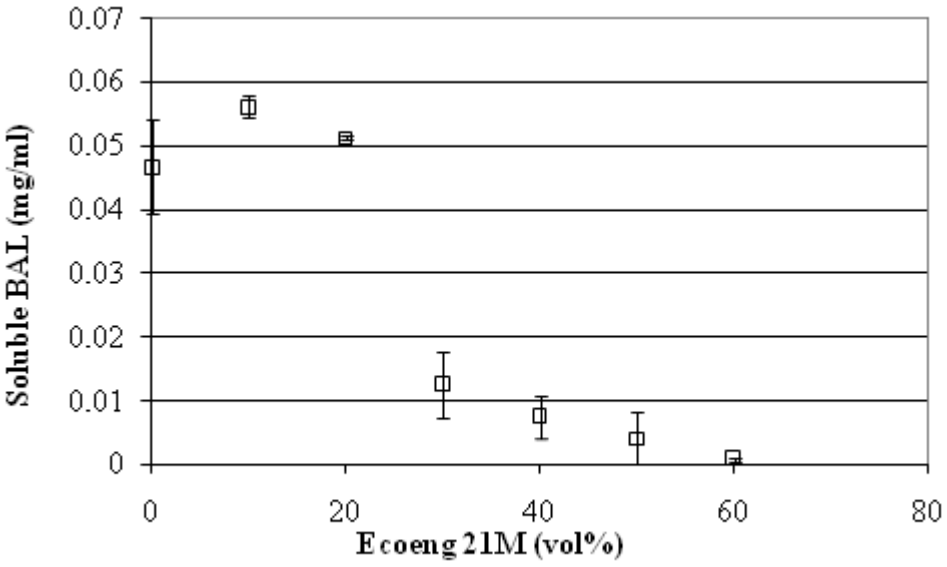


Figure 27: Solubility of BAL in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP, pH 6.5) and Ecoeng 21M. Soluble protein concentration determined after 1 h incubation and centrifugation at 13,000 for 5 min to remove the separate the precipitate. pH not adjusted.

III.1.2.1b. Enzyme stability in Ecoeng 21M/aqueous buffer mixtures

The stability of BAL in the presence of increasing concentrations of Ecoeng 21M was tested at 30°C and 40°C. At 30°C, there was no significant difference of half-life with 0, 20, and 40 vol% Ecoeng 21M (in all cases- about 5 days). Only in 60 vol% Ecoeng 21M the stability was decreased (**Fig. 28**).

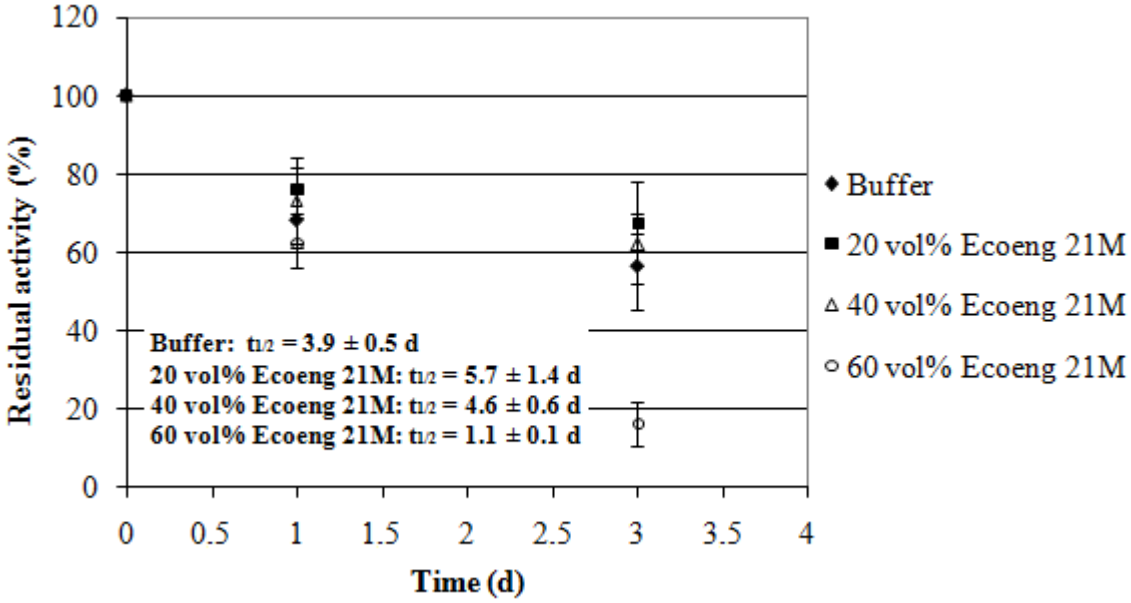


Figure 28: Stability of BAL in mixtures of 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) and Ecoeng 21M, 30°C. pH adjusted to 8. Error bars represent the mean of two measurements.

Unlike the addition of DMSO, 20 vol% Ecoeng 21M did not improve the stability of BAL at 40°C (**Fig. 29**).

Stability of BAL varied considerably among different lyophilisate preparations (compare half-life of BAL in buffer in **Fig. 17** and **28**). The resulting half-lives were reproducible only within one set of experiments; therefore it was necessary always to include control samples as a reference.

The stability of BFD*H281A* in Ecoeng 21M was tested only at 30°C. Apparently, any addition of Ecoeng 21M caused destabilization of the enzyme (**Fig. 30**). These results are not surprising and they partly explain the low activity of BFD*H281A* under the same conditions (**Fig. 26**).

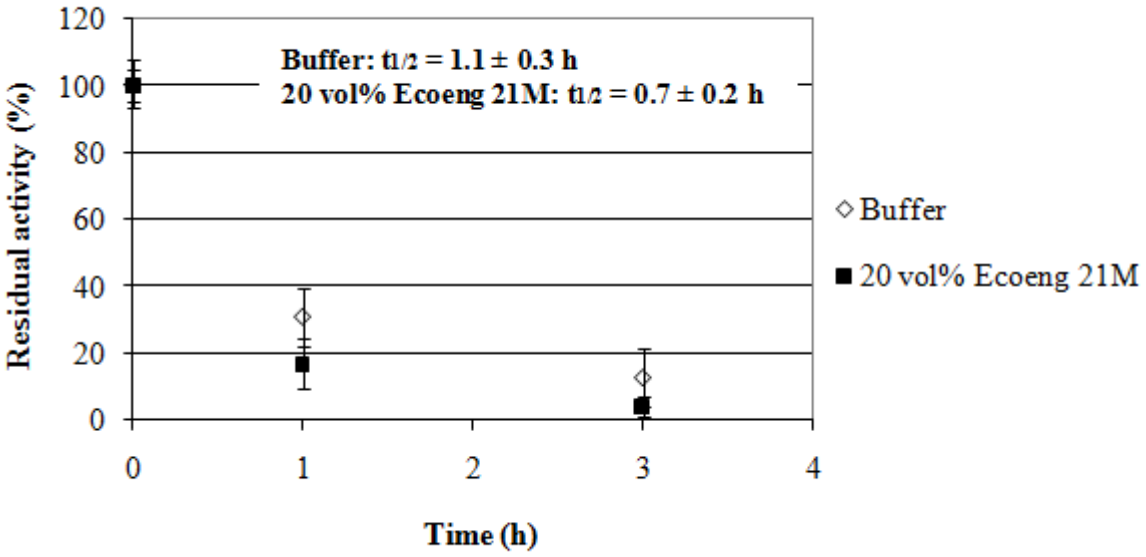


Figure 29: Stability of BAL in 50 mM potassium buffer (2.5 $MgSO_4$, 0.5 mM ThDP, pH 8) with and without 20 vol% Ecoeng 21M, 40°C. pH adjusted to 8. Error bars represent the mean of two measurements.

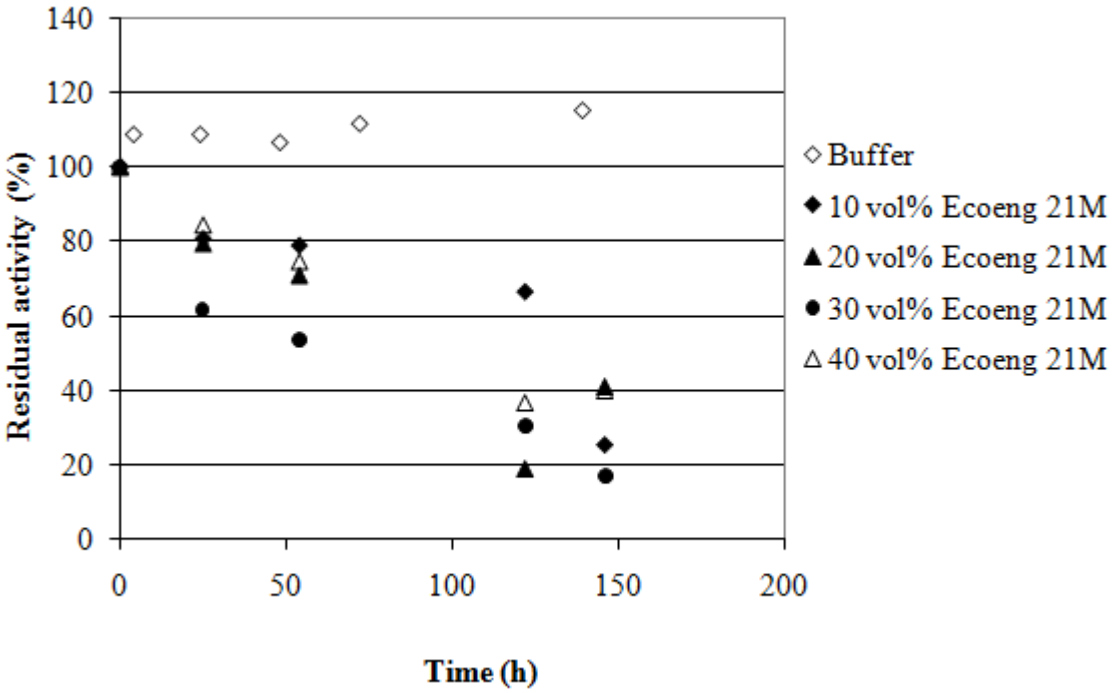


Figure 30: Stability of BFD*H281A* in mixtures of 50 mM potassium phosphate buffer (2.5 $MgSO_4$, 0.5 mM ThDP) and Ecoeng 21M, 30°C. pH adjusted to 6.5. Experiment was performed once to test the tendency.

III.1.2.2. Ecoeng 1111P as a cosolvent

In contrast to Ecoeng 21M, which influenced differently both enzymes, Ecoeng 1111P caused similar effects. With both enzymes, addition of small amounts of the ionic liquid led to a slight increase of activity, peaking at 20 and 10 vol% Ecoeng 1111P for BALand BFD*H281A*, respectively (**Fig. 31, 32**). Obviously, the inactivation of BAL at higher concentrations of Ecoeng 1111P was caused mainly by protein precipitation. For the activity of BFD*H281A*, the ionic liquid Ecoeng 1111P is a much better solvent than Ecoeng 21M, as it caused almost no precipitation and significantly lower inactivation compared to the effect of Ecoeng 21M (**Fig. 26**).

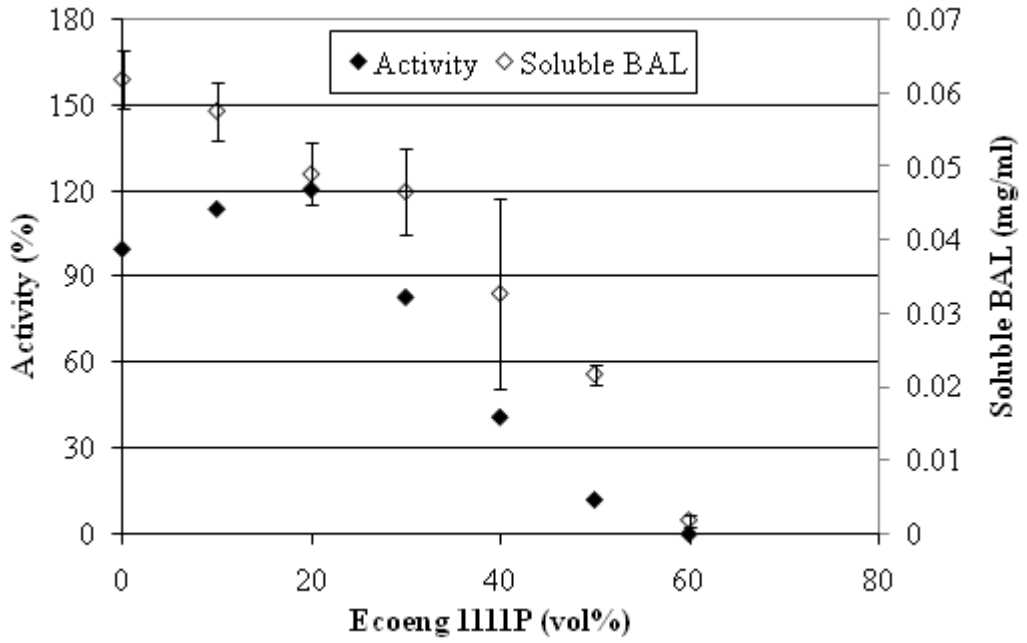


Figure 31: Initial reaction rates of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and Ecoeng 1111P. pH adjusted to 8. Activity measurements performed once, protein content is a mean value of three measurements.

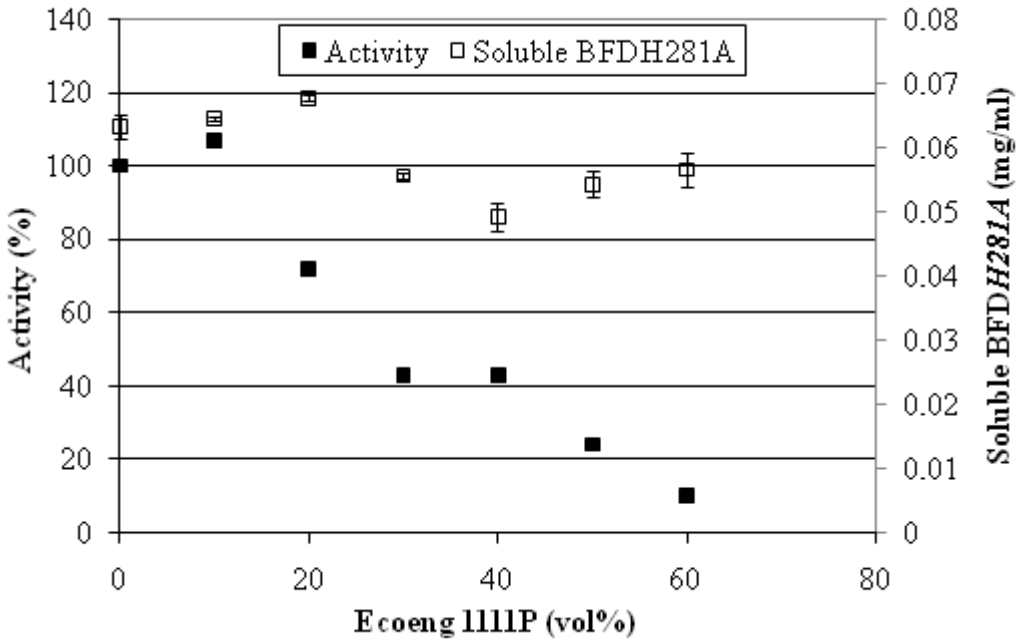


Figure 32: Initial reaction rates of BFD*H281A*-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and Ecoeng 1111P. pH adjusted to 6.5. Activity measurements performed once, protein content is a mean value of three measurements.

III.1.2.3. Other ionic liquids

The effect of 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIM.BF_4$) and EHMPES (1-ethyl-3-hydroxymethylpyridinium ethylsulfate) was investigated only on BAL. Although addition of 10 vol% $BMIM.BF_4$ caused a slight increase of activity, in 20 vol% $BMIM.BF_4$ it was already only 20% of the initial activity in buffer and in 40 vol% $BMIM.BF_4$ the enzyme was completely inactivated (**Fig. 33**). One possible reason for the inactivation in $BMIM.BF_4$ is the high viscosity of this IL, as the viscosity is one of the major solvent properties that affect enzyme activity in ILs, which can affect the activity due to lower diffusion rates (**Yang** and **Pan**, 2005; **Yang** et al, 2007; **van Rantwijk** and **Sheldon**, 2007) .

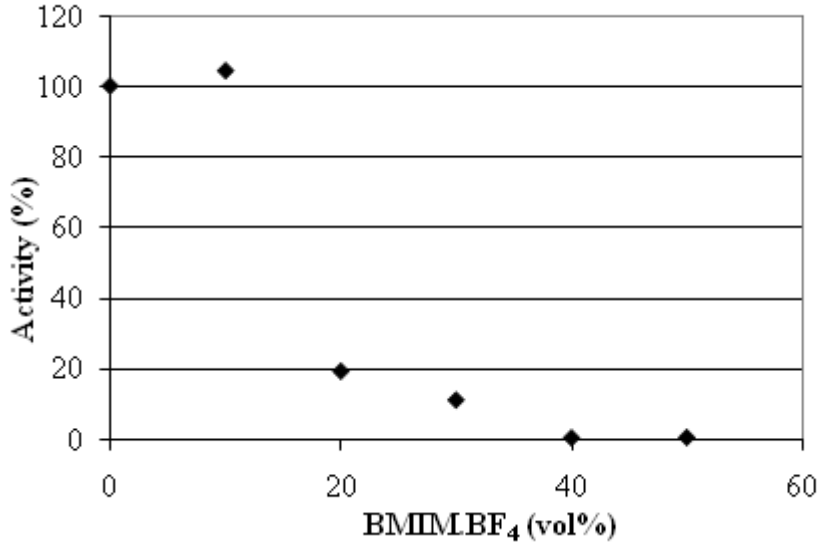


Figure 33: Initial reaction rates of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and $BMIM.BF_4$. pH adjusted to 8. Experiment was performed once to test the tendency.

Increasing concentration of EHMPES caused gradual inactivation of the enzyme and in 60 vol% EHMPES it was completely inactive (**Fig. 34**). Soluble enzyme concentrations were not measured.

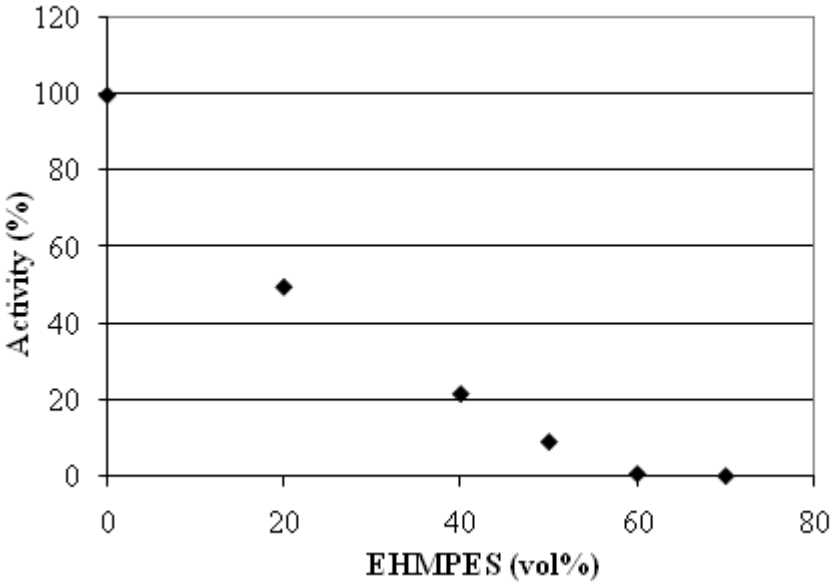


Figure 34: Initial reaction rates of BAL-catalysed benzoin formation in mixtures of 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) and EHMPES. pH adjusted to 8. Experiment was performed once to test the tendency.

III.1.2.4. Hofmeister series of ionic liquids

As the Hofmeister series of salts has an influence on the stability and activity of enzymes at moderate to high salt concentrations (**Broering** and **Bommarius**, 2005; **Introduction**, **I.2.1.2.**), the data from the intial rate measurements with BAL in 20 vol% (~1M) ILs were used to evaluate the effect of different ions: cosmotropic and chaotropic anions and cations.

The activity of BAL (**Fig. 35**) follows the Hofmeister series of ions of ILs which was deduced by **Zhao** et al. (2006[a]) based on their studies on hydrolases:

$PO_4^{3-} > citrate^{3-} > EtSO_4^{-} > CF_3COO^{-} > Br^{-} > BF^{4-}$

$(EMIM)^{+} > (BMIM)^{+} > (HMIM)^{+}$

Ecoeng 21M and Ecoeng 1111P (**Table 3**) contain stabilizing cations and they inproved the reaction rate compared to aqueous buffer by 144 and 121 %, respectively. In addition, Ecoeng 1111P contains the anion rated as the most stabilizing. EHMPES posesses the ethylsulfate anion which is considered as relatively good and its cation is of unknown influence, propably destabilizing, resulting in 50% of the activity in buffer. Finally, $BMIM.BF_4$ contains a neutral cation and a destabilizing anion and the enzyme showed only 20% activity compared to buffer (**Fig. 35**).

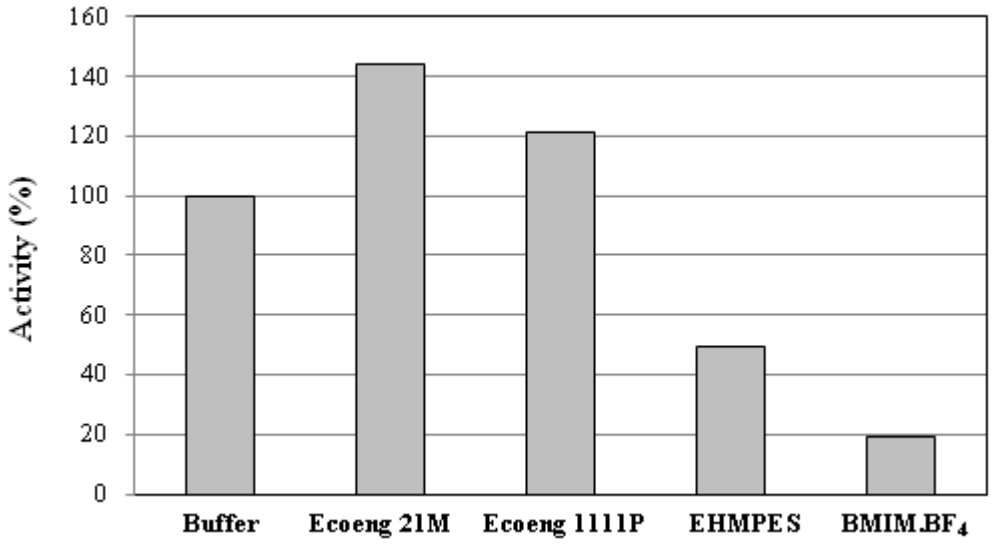


Figure 35: Activity of BAL in 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) with 20 vol% of different ionic liquids. pH adjusted to 8.

Unfortunately, the diethyleneglycolmonomethylethersulfate anion from Ecoeng 21M and the pyridinium-containing cation from EHMPES were not classified in the above mentioned study. However, the stabilizing effect of Ecoeng 1111P on both enzymes can be explained using the Hofmeister series as caused by an ionic liquid which contains both most stabilizing anion and cation.

With BFD*H281A*, only the effects of Ecoeng 21M and Ecoeng 1111P were tested; therefore the effects of ions could not be arranged according to Hofmeister series. The activity of BFD*H281A* in Ecoeng 1111P (IL rated as a salt with both stabilizing ions) was generally comparable to that with BAL, whereas the effect of Ecoeng 21M was worse than with BAL, most probably due to precipitation.

III.1.3. Studies on enzyme conformation in presence of organic solvents and ionic liquids

III.1.3.1. Investigation of enzyme unfolding by tryptophan fluorescence spectroscopy

The tryptophan fluorescence of BAL in presence of different concentrations of urea as a denaturating agent was studied previously (**Janzen**, 2002). It was found that in the presence of increasing concentrations of urea the emission maximum was shifted to higher wavelength: from 342 nm (100% catalytically active molecule) to 345 nm (catalytically inactive molecule) (**Fig. 36**). A further increase of the urea concentration caused a shift of the maximum emission wavelength to 351.5 nm. The red shift of the fluorescence maximum is a consequence of the exposition of aromatic residues (mainly tryptophan) from the hydrophobic protein interior to the solvent. The exact location of this maximum depends to some extend on the nature of the solvent, e.g. buffer species and concentration (**Schmid**, 1989). In hydrophobic environment the emission maximum is shifted to lower wavelength.

The unfolding diagram (**Fig. 36**) of BAL shows two steps. It was suggested that during the first step from 1 to 3.5 M urea dissociation of ThDP occurs, leaving the tetrameric enzyme intact. In the second step from 5 to 6 M urea the tetramer is dissociated into dimers and monomers followed by unfolding of the protein chains.

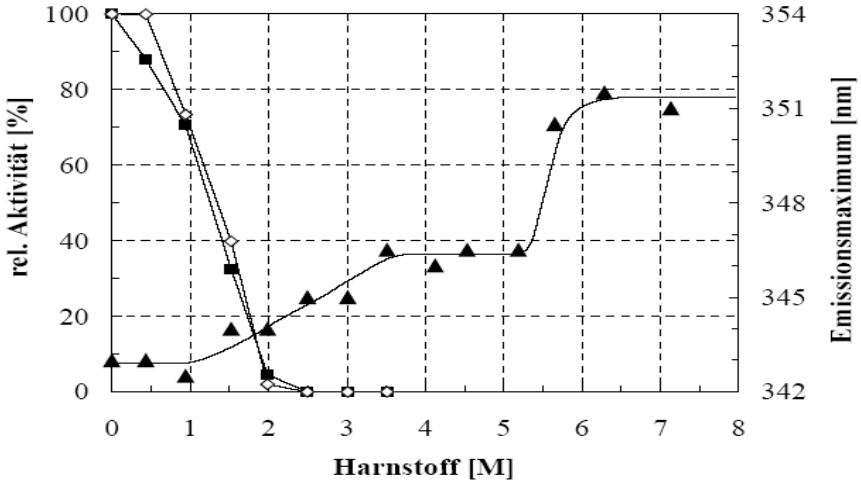


Figure 36: Unfolding diagram of BAL monitored by tryptophan fluorescence and residual activity in potassium phosphate buffer containing increasing concentrations of urea (from: Janzen et al., 2002).

In this thesis the effect of organic solvents and ionic liquids on the tryptophan fluorescence of BAL and BFD*H281A* was investigated. In the presence of increasing concentrations of organic solvents the emission maximum of both enzymes was shifted to lower wavelength (**Fig 37, 38**) along with decreasing activity. As was already shown (**Fig. 36**), the shift of enzyme conformation from native to the unfolded state in the presence of urea was accompanied by a red shift. Therefore, a blue shift of the emission maximum could be associated with lowered flexibility upon addition of organic solvents. Organic solvents are known to cause water-stripping which leads to an increase of enzyme rigidity (**Gorman** and **Dordick**, 1992; **Gupta**, 1992).

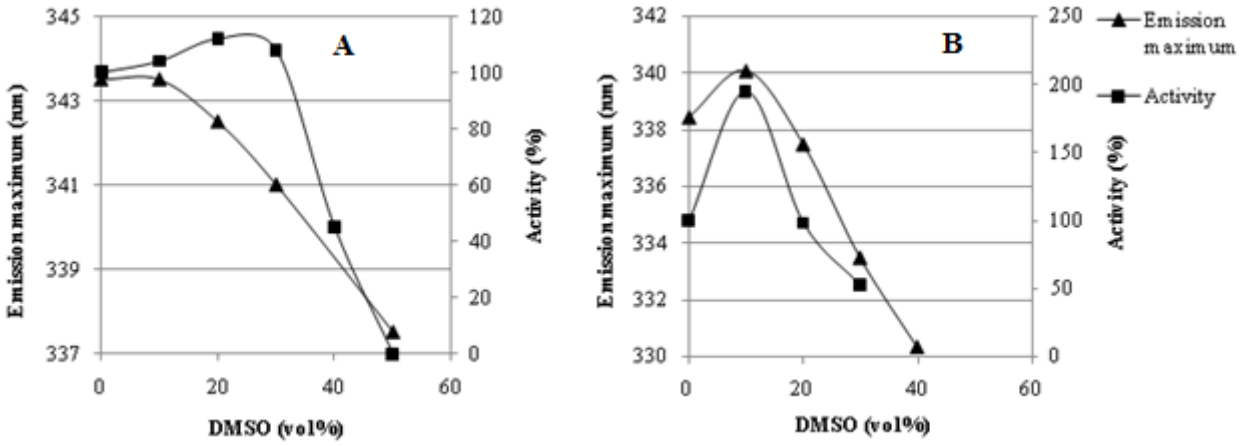


Figure 37: Tryptophan fluorescence and activity of BAL (A) and BFD*H281A* (B) in 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) containing increasing concentrations of DMSO. pH adjusted to 8 (BAL) and 6.5 (BFD*H281A*).

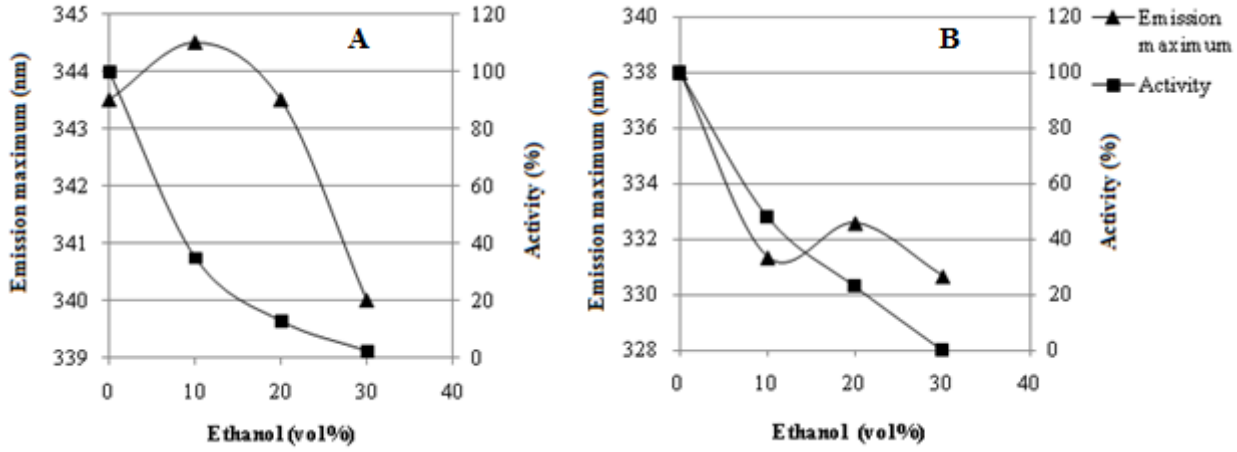


Figure 38: Tryptophan fluorescence and activity of BAL (A) and BFD*H281A* (B) in 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) containing increasing concentrations of ethanol. pH adjusted to 8 (BAL) and 6.5 (BFD*H281A*).

Examples of tryptophan fluorescence in the presence of increasing concentrations of acetone and 2-propanol given in **Appendix** (**Fig. A7**) follow the same trend.

In contrast to organic solvents, increasing concentrations of ionic liquids caused a more or less pronounced red shift of the emission maximum (**Fig. 39, 40**) indicating a process of unfolding as observed with urea (**Fig. 36**).

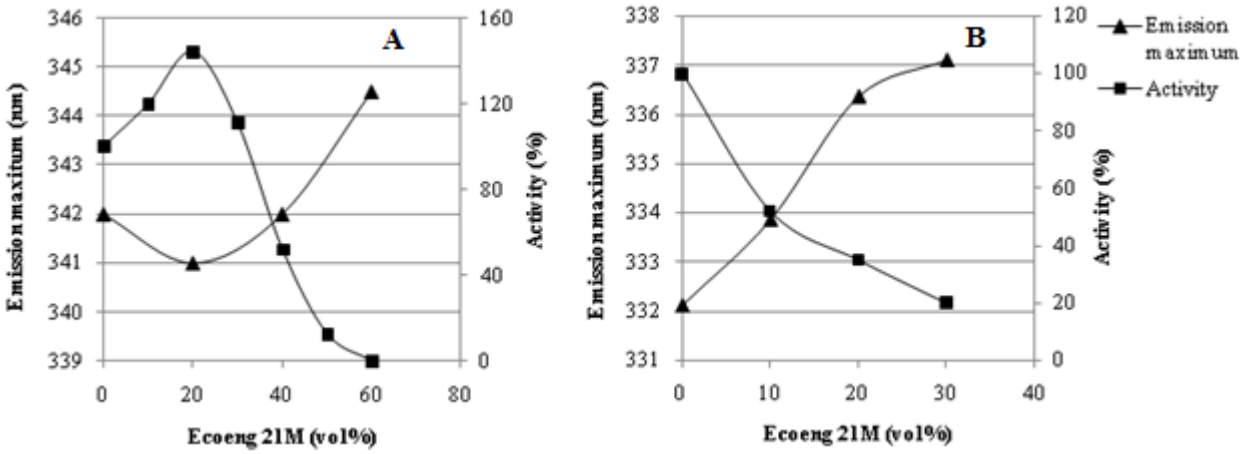


Figure 39: Tryptophan fluorescence and activity of BAL (A) and BFD*H281A* (B) in 50 mM potassium phosphate buffer containing increasing concentrations of Ecoeng 21M.

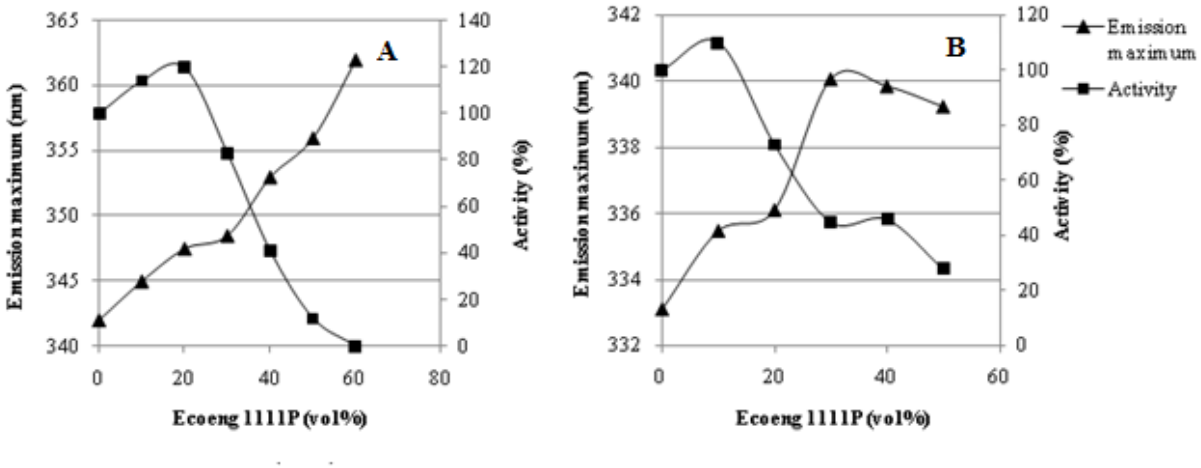


Figure 40: Tryptophan fluorescence and activity of BAL (A) and BFD*H281A* (B) in 50 mM potassium phosphate buffer containing increasing concentrations of Ecoeng 1111P.

Although the emission maxima were red-shifted in both ILs, the effect of Ecoeng 1111P was stronger (10 - 20 nm). This effect could be interpreted in terms of more pronounced enzyme unfolding in Ecoeng 1111P. However, as the shift of the emission maximum is dependent on the protein, the ions and their concentrations, it is not a measure for a defined folding/unfolding state.

Although the tryptophan fluorescence is enzyme- and solvent-dependent, the following common trends were observed:

- Upon addition of organic solvents the emission maximum was shifted to lower wavelength in all cases. This blue shift of tryptophan fluorescence is probably associated with a lower strucutal flexibility of the proteins, e.g. shrinking of the molecule and water-stripping.

- With increasing concentrations of ionic liquids the emission maximum of the tryptophan fluorescence was generally shifted to higher wavelength although the absolute values were different for both ionic liquids. Red shift suggests a progressive unfolding of the enzyme, as confirmed by the denaturation effect of urea (**Fig. 36**).

- In all cases, the addition of solvents caused an increase of absorption intensity. Control studies on free tryptophan showed that the presence of solvents affected the intensity but did not affect the emission maximum significantly.

All the experiments were performed twice with a very good reproducibility (±1.5 nm).

III.1.3.2. Investigation of the helical content by CD spectroscopy

The CD spectra of proteins can provide information about their secondary and tertiary structure. The determination of the ellipticity at 222 nm is of major interest because this is the wavelength where α-helices absorb. An α-helix is characterized by a negative elipticity (measured in mdeg) which disappears upon unfolding.

As a control for these experiments, complete unfolding by high concentrations of urea was followed. The ellipticity of BAL (0.5 mg/ml) treated with 8 M urea approached 0 mdeg within one minute of incubation. As a more stable enzyme in general, BFD*H281A* (0.5 mg/ml) showed a slower unfolding process, reaching complete unfolding after ~30 min of incubation (**Fig. 41**).

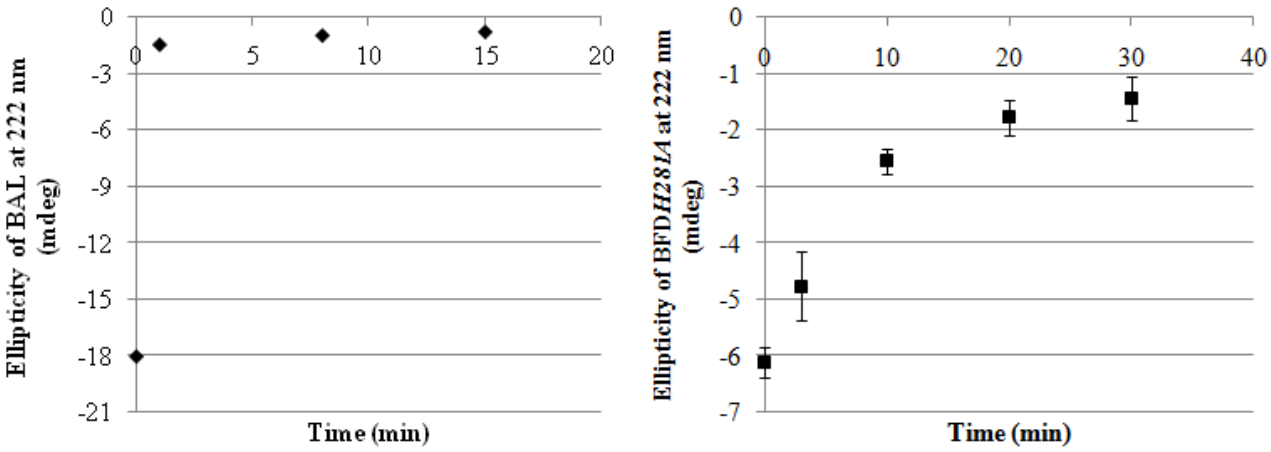


Figure 41: Time course of urea-induced unfolding of BAL and BFD*H281A*, followed by CD at 222 nm in 8M urea. Protein concentration 0.5 mg/ml. Ellipticity of BAL measured once. Error bars indicate SD of two independent measurements of BFH*281A* ellipticity.

In contrast to urea, increasing concentrations of organic solvents did not unfold the enzymes (**Fig. 42**, **43**). Both enzymes were initially incubated in organic solvents for 5 min because this was approximately the time used in the activity assays. As no significant change of ellipticity occurred (**Fig. 42**), the experiment was repeated after an incubation of 20 h. However, decreased ellipticity was observed only with BAL in higher concentrations of ethanol. Once again, BFD*H281A* showed a better tolerance to solvents with no change

of ellipticity after 20 h incubation in ethanol (**Fig. 43**). Similar results with acetone as a cosolvent are shown in **Appendix** (**Fig. A8**).

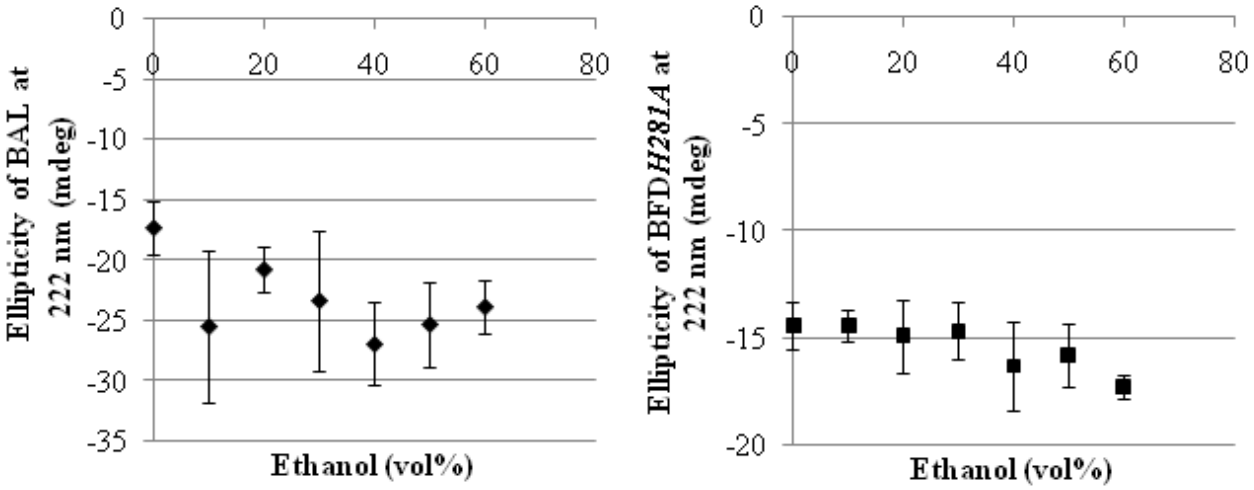


Figure 42: Ellipticity of BAL and BFD*H281A* in 50 mM potassium phosphate buffer (2.5 mM MgSO4, 0.5 mM ThDP) containing increasing concentrations of ethanol after 5 min incubation at 30°C. pH adjusted to 8 (BAL) and 6.5 (BFD*H281A*). Protein concentration 0.5 mg/ml. Error bars indicate SD of two independent experiments.

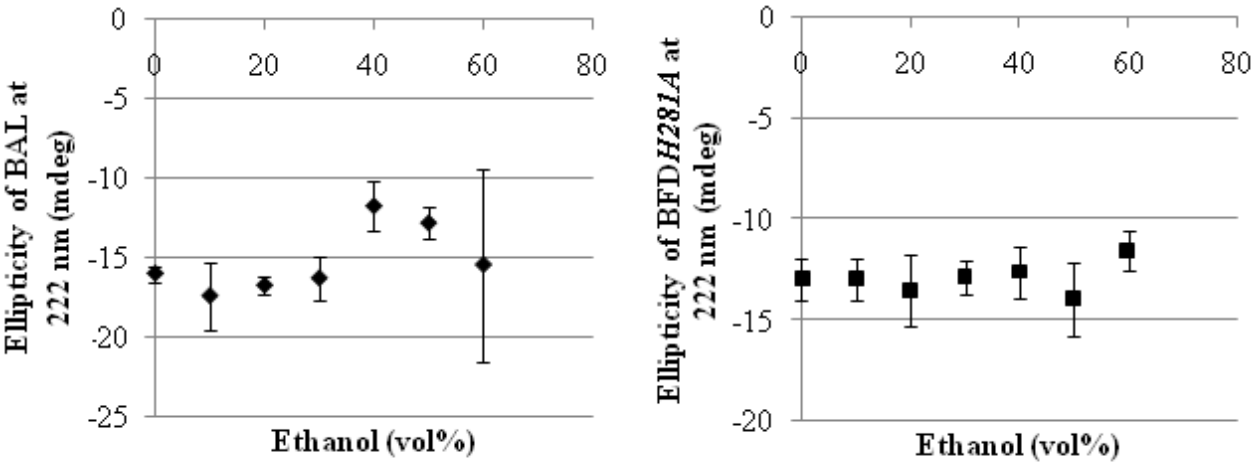


Figure 43: Ellipticity of BAL and BFD*H281A* in 50 mM potassium phosphate buffer (2.5 mM MgSO4, 0.5 mM ThDP) containing increasing concentrations of ethanol after 20 h incubation at 30°C. pH adjusted to 8 (BAL) and 6.5 (BFD*H281A*). Protein concentration 0.5 mg/ml. Error bars indicate SD of two independent experiments.

The CD spectroscopy data confirmed the results from the fluorescence studies, demonstrating that the progressive enzyme inactivation in the presence of water-miscible organic solvents (ethanol and acetone) is not due to severe unfolding of the structure.

Unfortunately, this method could not be used for DMSO and ionic liquid due to signal interference in the range of 222 nm.

III.2. Activity and stability of BAL and BFD*H281A* in water-free media

III.2.1. Effect of pure organic solvents and ionic liquids

III.2.1.1. Activity of BAL in pure organic solvents and ionic liquids

The initial experiments in pure organic solvents were intended to obtain first results on activity of BAL in these media, since no previous data about such systems were available. It was found that the enzyme is capable of very slow benzoin production in 100 vol% petroleum ether, hexane and hexadecane (**Fig. 44**). In the other tested water-immiscible solvents – toluene and MTBE, as well as in water-miscible solvents DMSO, ethanol and acetone, and ionic liquids Ecoeng 21M and Ecoeng 1111P – no product was detected even after 2 weeks. The enzyme concentration in all solvents was 270 μg/ml (55 times more than in the standard assay in potassium phosphate buffer) and it was added as a solid lyophilisate. The enzyme activity was closely correlated with the log P-value of the solvents (**Fig. 45)**. The activity of BAL in pure organic solvents follows the rule ***the higher the log P the better***, as described in **I.2.2.1**. The activity of BFDH*281A* was tested only in pure water-miscible solvents and ionic liquids (DMSO, acetone, and ethanol, Ecoeng 21M and Ecoeng 1111P) and like with BAL no product formation was observed.

Both enzymes were completely insoluble in the tested systems and formed emulsions.

The activity of BAL in pure water-immiscible solvents was initially tested with unknown water activity. The amount of water in the solvents was assumed to be 0, as only freshly opened bottles were used, except for the petroleum ether. The latter may be an explanation for the higher activity of BAL in this solvent compared to hexadecane. Presence of small amounts of water in water miscible solvents does not increase the water activity. However, in water immiscible solvents traces of water increased the water activity significantly. This is why the experiment without knowledge of water activity was only

intended as a first check to find out whether BAL was able to catalyse the reaction in water-free media.

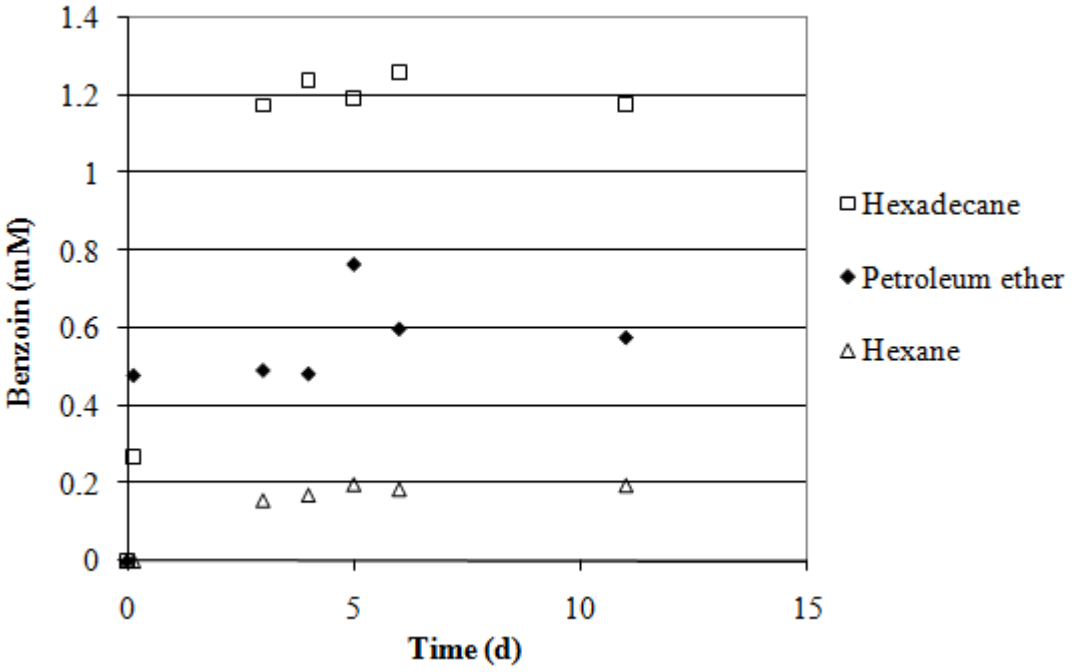


Figure 44: Benzoin formation catalysed by BAL (270 µg/ml) in 100 % water immiscible solvents, containing 0.54 mM ThDP, 2.7 mM $MgSO_4$, with 10 mM benzaldehyde.

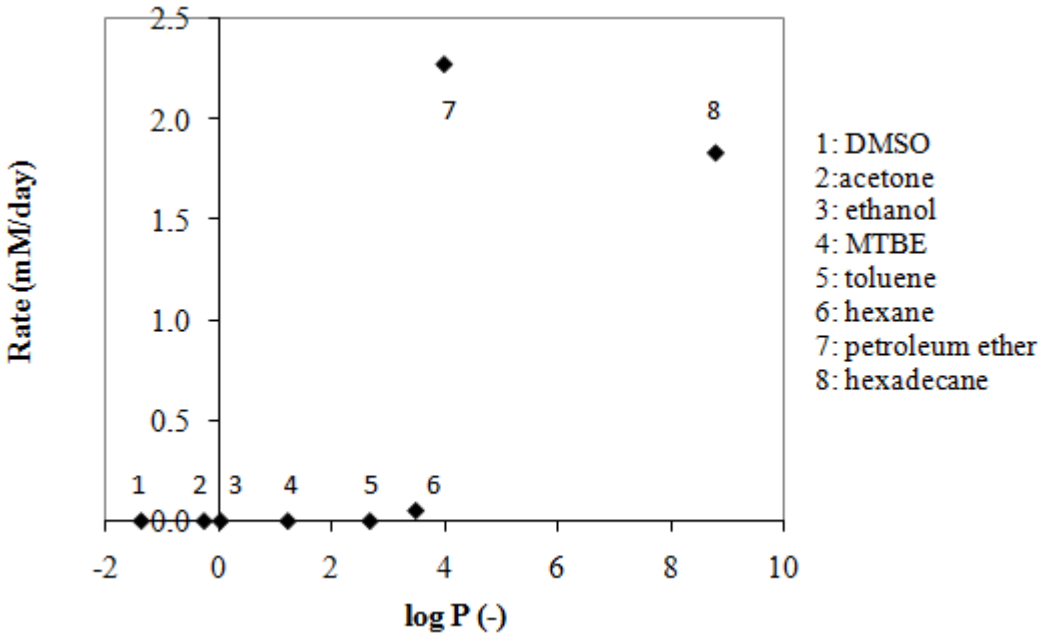


Figure 45: Dependence of reaction rate on solvent log P- value. Best activity in log P > 4.

The enzyme, the substrate and the solvents were put separately in desiccators over concentrated salt solutions and left for 24 hours in order to fix the water activity. Equilibration of the systems was not monitored. The activity of BAL was measured at a_w 0.12 (LiCl), 0.84 (KCl) and 0.97 (K_2SO_4) (**Valivety** et al., 1992[a]; **Bell** et al, 2001) in petroleum ether with and without additional cofactor because larger amount of cofactors could be required under the conditions applied (see **III.3.1**). The following results were obtained:

1. At water activity of 0.12 no reaction was observed both with and without cofactors.

2. At water activity of 0.84 a rate of about 0.05 mM benzoin/day was observed in petroleum ether without additional cofactors. This activity was 200,000 times lower than in 50 mM potassium phosphate buffer, containing 0.5 mM ThDP and 2.5 mM $MgSO_4$. In the reaction mixture with added cofactors no product was formed. ThDP and $MgSO_4$ cannot be dissolved in petroleum ether, so they form aggregations together with the enzyme, which is probably the main reason for the poor activity of the enzyme in water-free systems.

3. At water activity of 0.97 only 0.7 mM product was formed after 4 days in the reaction mixture without cofactors. Again, adding solid cofactors caused inactivation.

The enzyme and the ThDP were practically insoluble in pure organic solvent, which is the most important reason for the poor enzyme activity under the tested condition. In addition, tThis very low activity in pure water-immiscible organic solvents could be a consequence of the reaction mechanism, which requires proton transfer.

III.2.1.2. Stability of BAL and BFD*H281A* in pure organic solvents

In water-miscible organic solvents and ionic liquids both enzymes were completely deactivated immediately and irreversibly after contact with the pure solvents and no residual activity was observed. In contrast to water-miscible solvents, BAL showed certain residual activity in pure water-immiscible solvents like petroleum ether, MTBE and hexadecane (**Fig. 46, 47**), although lower than in 50 mM potassium phosphate buffer containing 2.5 $MgSO_4$ and 0.5 mM ThDP. The reasons for this difference is that the water-miscible solvents, compared to water-immiscible solvents, provide harsher

conditions for the protein as they can strip bound water from it (**Yang** et al., 2004). The stability of BFD*H281A* was measured only in pure water-miscible solvents.

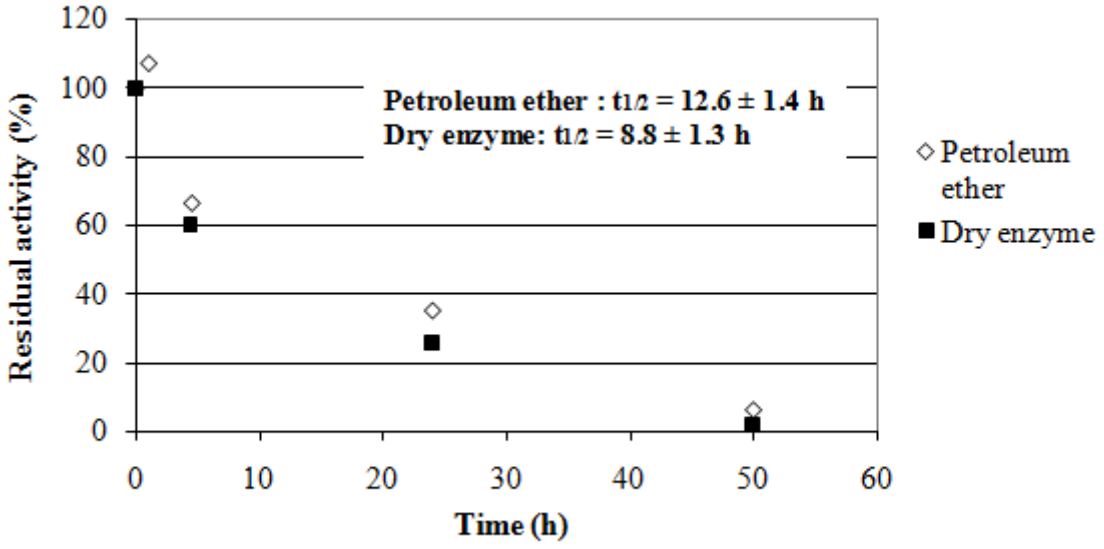


Figure 46: BAL stability in petroleum ether and without solvent (dry lyophilisate) at 30°C. BAL (270 µg/ml) was incubated in pure petroleum ether or kept in dry form (powder). The enzyme was isolated by centrifugation and dissolved in 50 mM potassium phosphate buffer (2.5 mM MgSO4, 0.5 mM ThDP, pH 8). Residual activity was assayed using benzoin formation assay by HPLC.

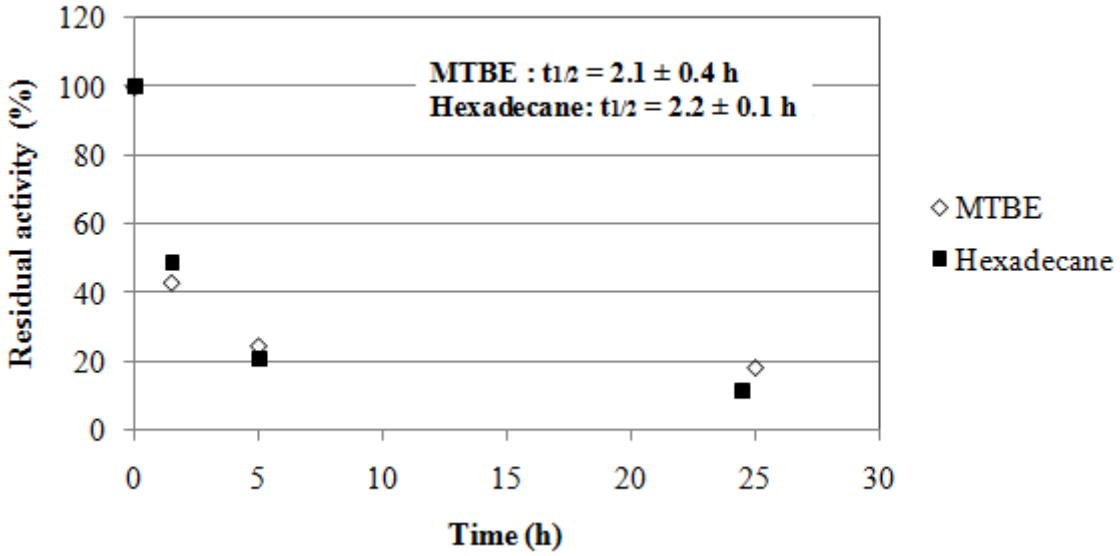


Figure 47: BAL stability in MTBE and hexadecane at 30°C. For details see legend under Fig. 46.

The highest stability of BAL was found in 100 % petroleum ether (half-life 12.6 h) (**Fig. 46**). This result correlates also with highest activity in this solvent, too (**Fig. 45**). In

both 100 % MTBE and hexadecane the stability of BAL was similar (half-life ~2 h) (**Fig. 47**). Furthermore, experiments to test the stability of a dry lyophilisate at 30°C were conducted and the half-life without solvent was 8.8 h (**Fig. 46**).

III.2.2. Effect of supercritical carbon dioxide

The carboligase activity of BAL and BFD*H281A* was tested under supercritical conditions in an autoclave (40°C, 100 bar). After 48 hours, no benzoin was formed with BAL. With BFD*H281A*, only traces of product were detected. This small difference is due to the better stability of BFD*H281A* at 40°C (see **III.1**) which allows the reaction to continue longer.

An important factor to be considered is the water activity in the autoclave. It was previously found that optimum water activity is crucial for the enzyme activity in a gas-phase reactor (**Mikolajek** et al., 2007). Optimum a_w was 100 % for BAL; whereas for BFD it was lower: 50 %. The estimated water activity in the autoclave was 0.5-0.7 which was closer to the optimum for BFD, supposedly also for BFD*H281A*. The lack of activity in supercritical CO_2 was not surprising, as the enzymes showed a very low activity in 100 vol% water-immiscible solvents (another water-free system), too.

To explain these results, the stability of both enzymes was tested in supercritical CO_2 (40°C, 100 bar). The half-life of BAL and BFD*H281A* were, 0.6 h and 173 h, respectively (**Fig. 48**). The shortest incubation time possible is 1 hour because certain time is needed before the systems gets into equilibrium; therefore the exact half-life of BAL can only be estimated.

The obtained results are comparable with the stability data at 40°C in potassium phosphate buffer (**III.1**), indicating that the most important parameter for the enzyme stability under supercritical conditions is the temperature.

The better thermostabilty of BFD*H281A* was supported by circular dichroism spectroscopy measurements of enzyme **melting point** (T_m). The melting point as a measure of thermostability is the temperature at which the enzyme loses 50% of its helical structure. The melting curves in **Fig. 49** show that both enzymes unfold upon thermal treatment, with BFD*H281A* again proving a higher thermostability. BAL starts losing its

helical structure more rapidly and shows a lower T_m (47.0 ± 1.5°C), whereas BFD*H281A* as a more thermostable enzyme has a higher T_m (62 ± 2.0°C).

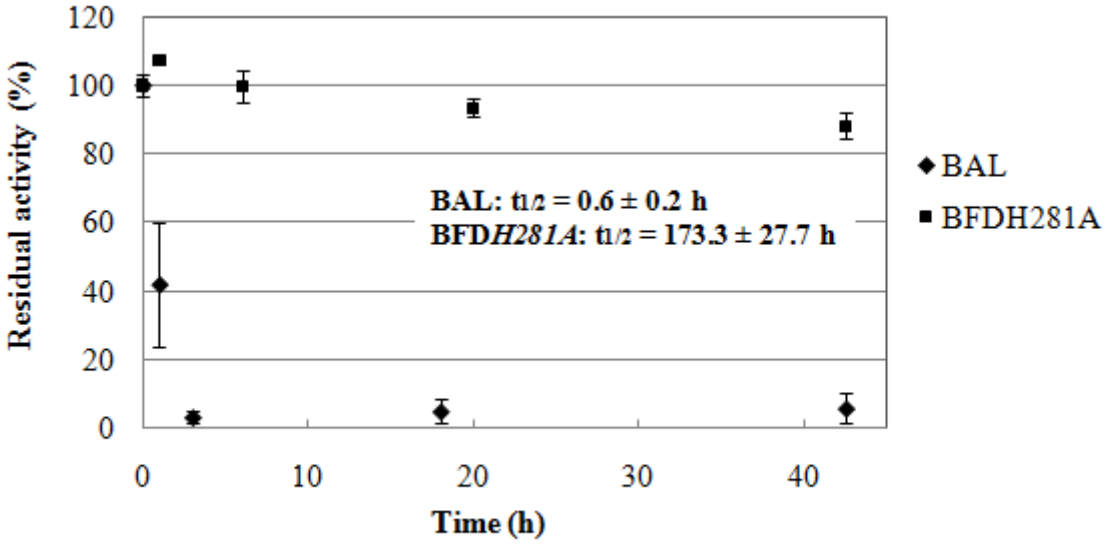


Figure 48: Enzyme stability under supercritical conditions (CO_2, 40°C, 100bar). The enzymes (50 µg) were incubated in dry form (powder). After different time intervals the probes were dissolved in 50 mM potassium phosphate buffer (pH 8 for BAL and 6.5 for BFD*H281A*). The residual activity was measured using the benzoin cleavage assay.

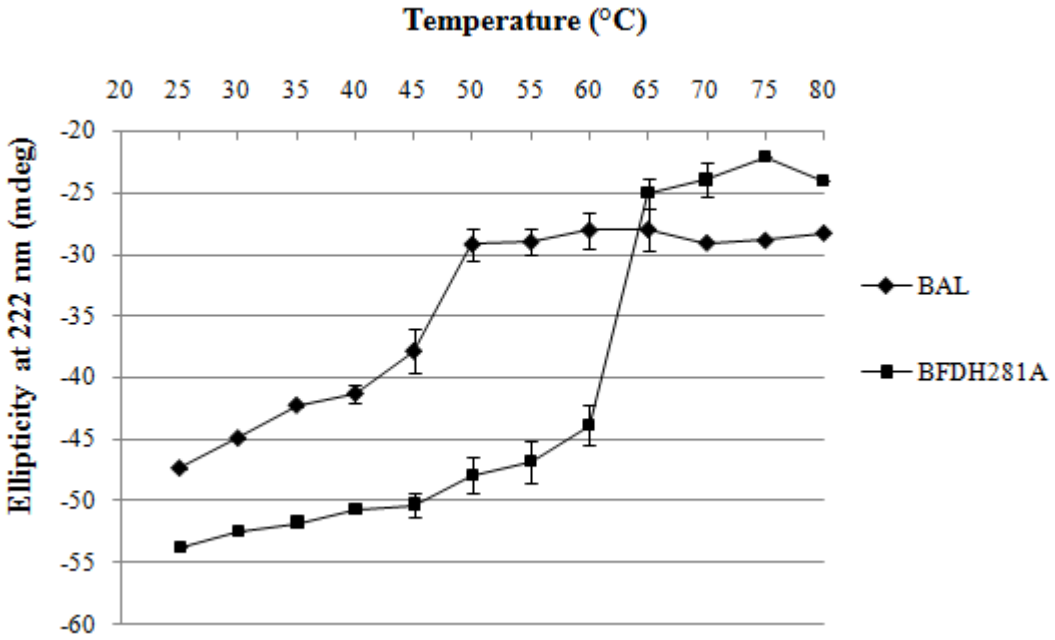


Figure 49: BAL and BFD*H281A* melting points. The enzymes BAL (0.8 mg/ml) and BFD*H281A* (0.7 mg/ml) were dissolved in 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP. Heating rate: 1°C/min; measurements: every 5 min. T_m: BAL (47.0 ± 1.5°C) and BFD*H281A* (62 ± 2.0°C).

III.3. Role of cofactor

III.3.1. Cofactor stability and requirement

BAL was almost not active in aqueous buffer without addition of 0.1-0.5 mM ThDP, although the cofactor amount in the lyophilisate was in large stoichiometric excess. This is an indication for either deactivated cofactor during freezing/drying or insufficient cofactor concentration in the reaction medium.

To investigate the reason for the enzyme deactivation in potassium phosphate buffer without additional cofactor, three differently treated samples of 50 mM potassium phosphate buffer containing 0.5 mM ThDP (lyophilized, frozen, and kept in fridge as a control) were tested as reaction media, using BAL as a catalyst. The activity was equal in the differently treated buffers indicating that no degeneration of the cofactor ThDP occurs during lyophilisation (freezing/drying). This experiment showed that not the freeze/drying but the insufficient amount of cofactor is the reason for inactivation of BAL.

Using the standard purification protocol (**II.4.1)**, the amount of ThDP after the desalting step is ~ 0.001 mM/µg BAL (or 1 µM ThDP/0.85 nM active sites), which is insufficient for activity although is stoichiometically enough to saturate the active sites. Therefore, excess cofactor (0.1-0.5 mM ThDP) has to be added to the aqueous buffer but in case of water-free media the cofactor cannot be provided in this way. In this experiment the enzyme lyophilisate was prepared with 100-fold higher amount of ThDP (0.1 mM/µg BAL), redissolved in 50 mM potassium phosphate buffer without ThDP and assayed using the benzoin formation assay by HPLC (**II.4.4.4**). As shown in **Fig. 50**, the activity was identical with the control sample (BAL lyophilised according to the standard protocol assayed in 50 mM potassium phosphate buffer with 0.5 mM ThDP). Therefore, the concentration required for maximum activity is much higher than the one needed to saturate the cofactor binding site.

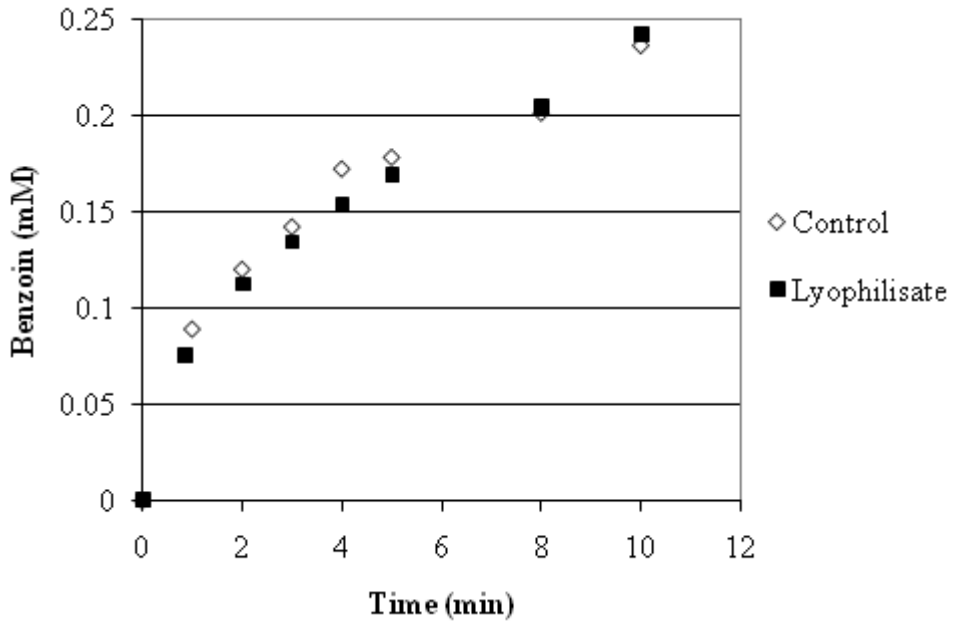


Figure 50: BAL-catalyzed benzoin formation in 50 mM potassium hosphate buffer with and without cofactors. Control: buffer containing 0.1 ThDP and 2.5 mM $MgSO_4$, BAL co-lyophilised with 0.001 ThDP/µg protein. Lyophilisate: buffer without cofactor, BAL co-lyophilised with 0.1 mM ThDP, 0.6 mM $MgSO_4$/µg protein).

The lyophilisates prepared with 0.1 mM ThDP/µg BAL were used in experiments, conducted in the absence of aqueous buffer or water, i.e. pure water-free media (organic solvents, $scCO_{2)}$ to make sure that there are enough cofactors in the reaction media (**III.2**).

III.3.3. Cofactor dissociation

As already shown, the non-conventional media affect the enzyme activity and stability to a large extend. These changes can be partly due to altered interactions between the enzyme and the cofactor molecules affected by the solvent properties, as ThDP is non-covalently bound to the active sites of BAL and BFD. Therefore, studying the effect of cosolvents on the cofactor binding can contribute to a better understanding of the reasons these changes occur.

In this experiment, the enzymes were dissolved in 50 mM potassium phosphate buffer containing different ThDP concentrations and constant Mg^{2+} concentration (2.5 mM). 0.02 mM was the lowest concentration possible since the lyophilisates contained 0.001 mM ThDP/µg BAL and 20 µg BAL were used in this assay (**II.4.4.3)**. At given time intervals

their residual activity was measured and compared with the residual activity of enzyme samples incubated in buffer/DMSO mixture (30 vol% DMSO). To follow the cofactor dissociation, it is very important to measure the residual activity in the same ThDP concentrations as in the incubation buffer because the active sites get saturated rapidly (see **Fig. 55**), whereas the activity assay is 1-1.5 min. Additional studies with various Mg^{2+} concentrations showed this cofactor did not influence the residual activity under the tested conditions.

In 50 mM potassium phosphate buffer containing low amounts of ThDP (0.020-0.034 mM), the enzyme got rapidly inactivated until reaching an equilibrium level (**Fig. 51, 52**). Interestingly, the activity of the enzyme incubated in 30 vol% DMSO remained constant. Upon increasing cofactor concentration up to 0.038 mM the difference between the initial and the final activity in buffer became smaller (**Fig. 53**).

Finally, the ThDP concentration became sufficient (0.12 mM) to keep constant activity for at least 250 min (**Fig. 54**). By contrast, in all samples containing 30 vol% DMSO the activity remained unchanged independent on the cofactor concentration. To prove that the inactivation was due only to loss of cofactor, buffer containing 0.12 mM ThDP was added to a sample incubated for 1 hour in buffer without additional cofactor, which resulted in an immediate recovery of 90% of the initial activity (**Fig. 55**).

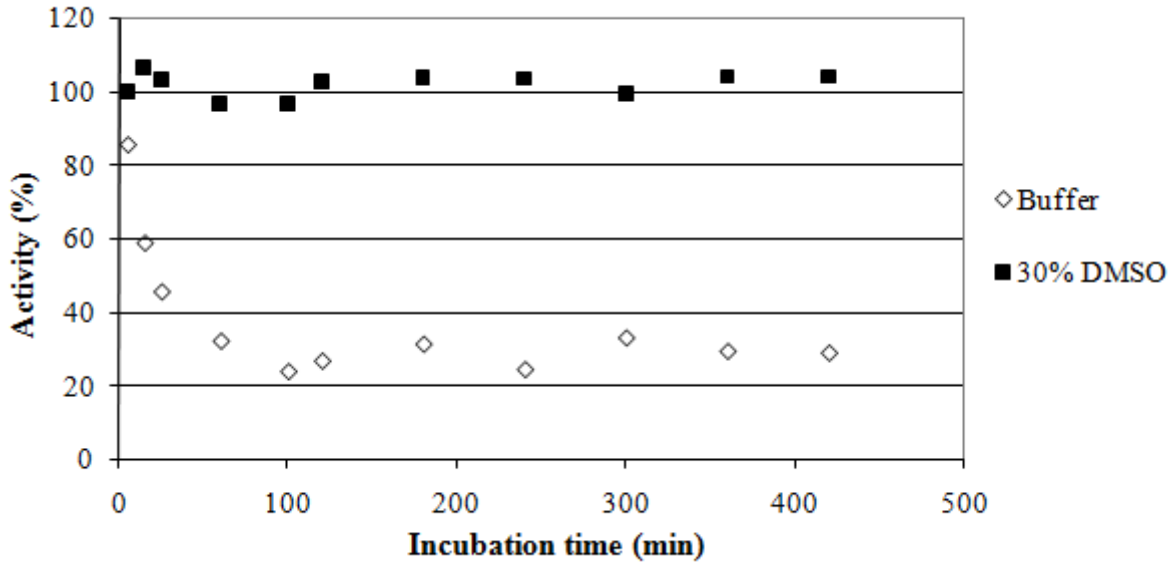


Figure 51: Residual activity of BAL incubated with 0.02 mM ThDP (and 2.5 mM $MgSO_4$) in the presence and absence of 30 vol% DMSO in potassium phosphate buffer, 30°C.

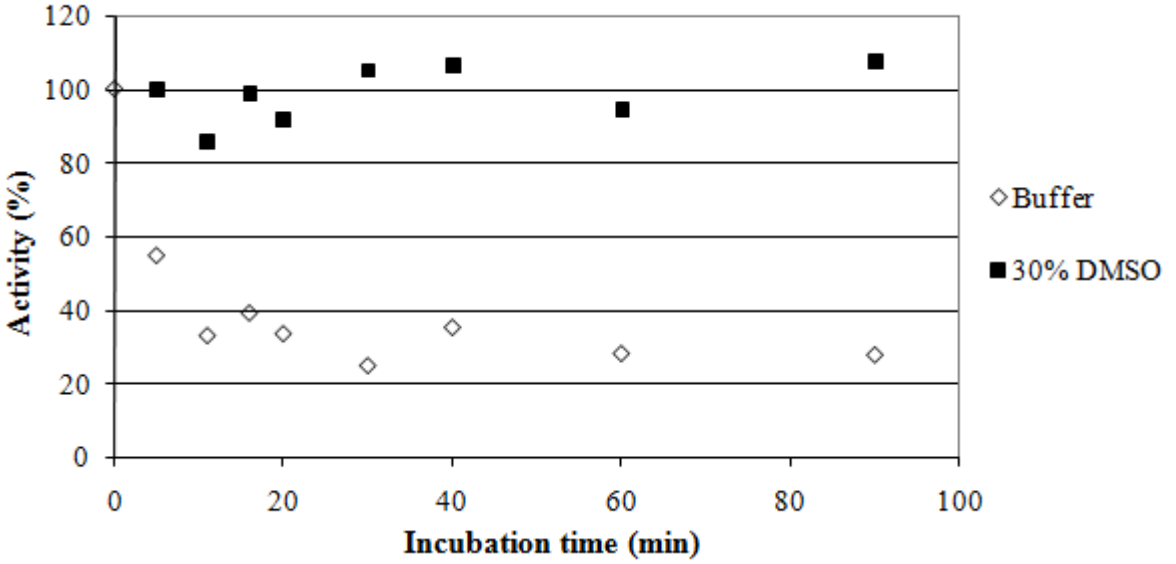


Figure 52: Residual activity of BAL incubated with 0.034 mM ThDP (and 2.5 mM $MgSO_4$) in the presence and absence of 30 vol% DMSO in potassium phosphate buffer, 30°C.

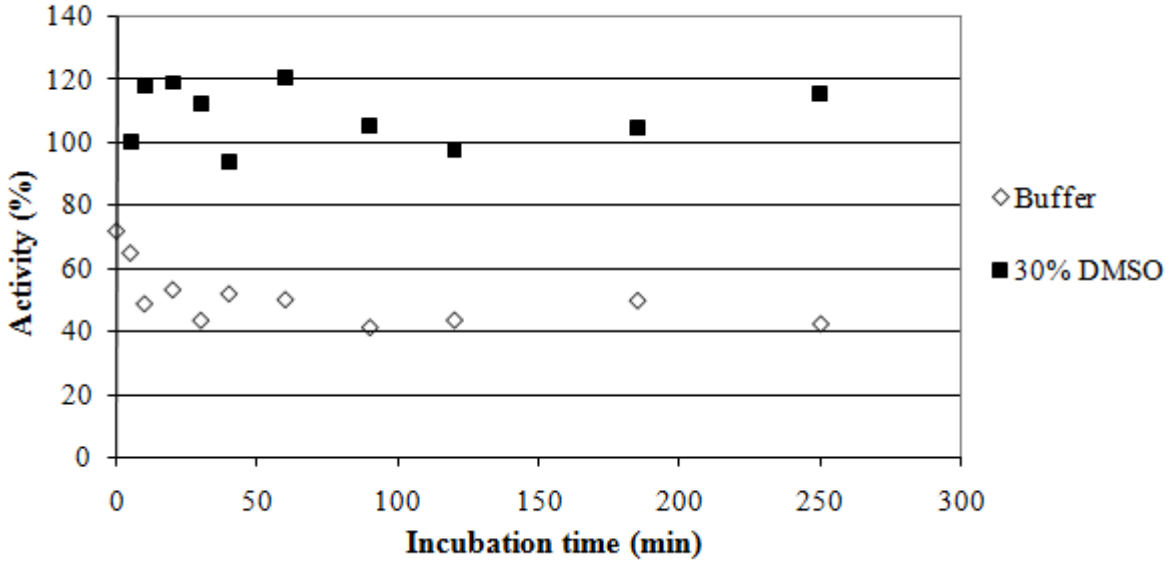


Figure 53: Residual activity of BAL incubated with 0.038 mM ThDP (and 2.5 $MgSO_4$) in the presence and absence of 30 vol% DMSO in potassium phosphate buffer, 30°C.

The studies shown in **Fig. 51-55** were performed at two different pH-values: phosphate buffer, pH 8, and 30 vol% DMSO containing solution, pH 8.8. In order to find out whether the pH is relevant for the observed differences in cofactor stability, the studies in 30 vol% DMSO were repeated at pH 8. As demonstrated in **Fig. 56**, the alteration of the

pH did not change the results compared to **Fig. 51**. Moreover, a similar result was obtained when the cosolvent DMSO was replaced with Ecoeng 21M. Like in DMSO, in 30 vol% Ecoeng 21M omitting the cofactor, the enzyme activity remained unchanged for at least 60 min (**Fig. 57**).

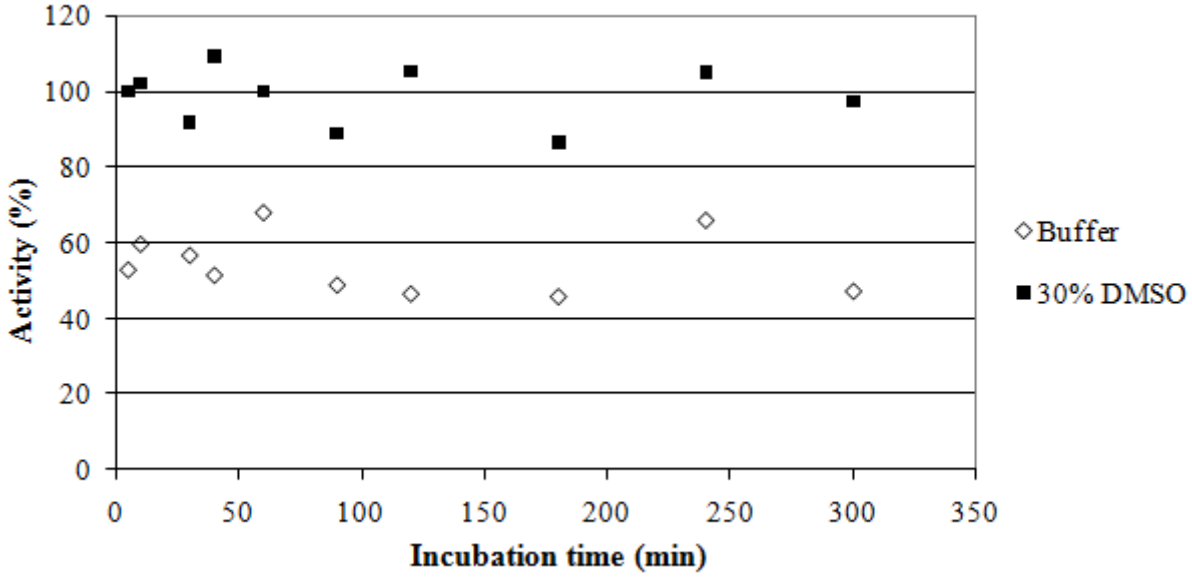


Figure 54: Residual activity of BAL incubated with 0.12 mM ThDP in the presence and absence of 30 vol% DMSO in potassium phosphate buffer, 30°C.

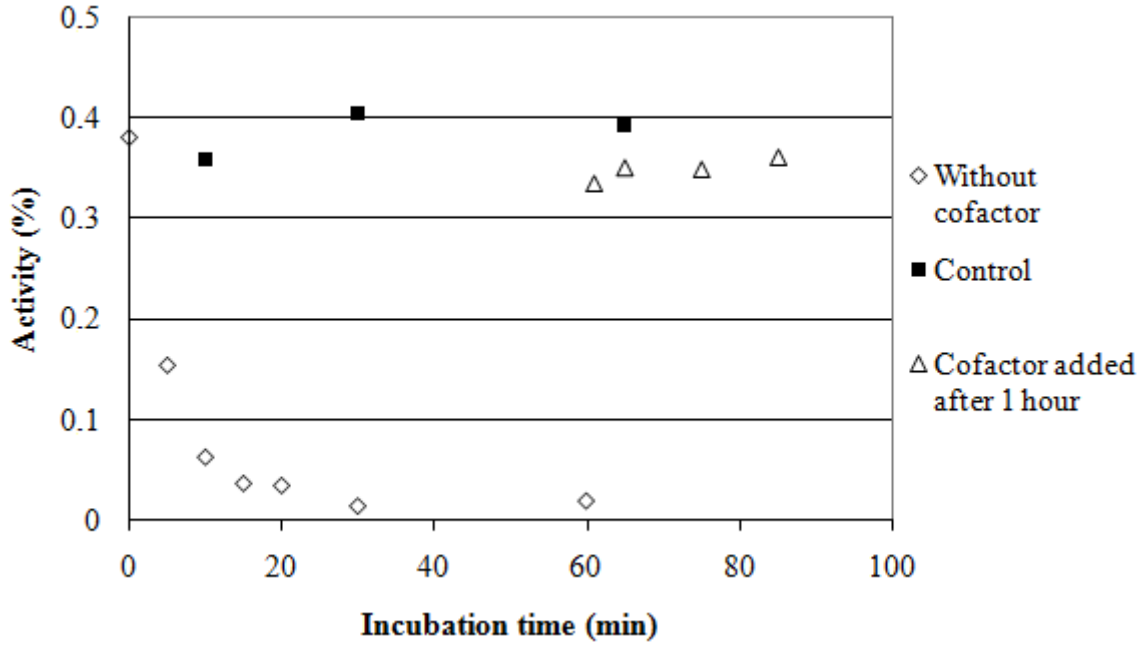


Figure 55: Cofactor dissociation reversibility. Residual activity of BAL measured in: 50 mM potassium phosphate buffer containing 0.02 mM ThDP, 2.5 mM $MgSO_4$ (without additional ThDP); 50 mM potassium phosphate buffer containing 0.5 mM ThDP (control) and 50 mM potassium phosphate buffer with 0.5 mM ThDP added after 1 h.

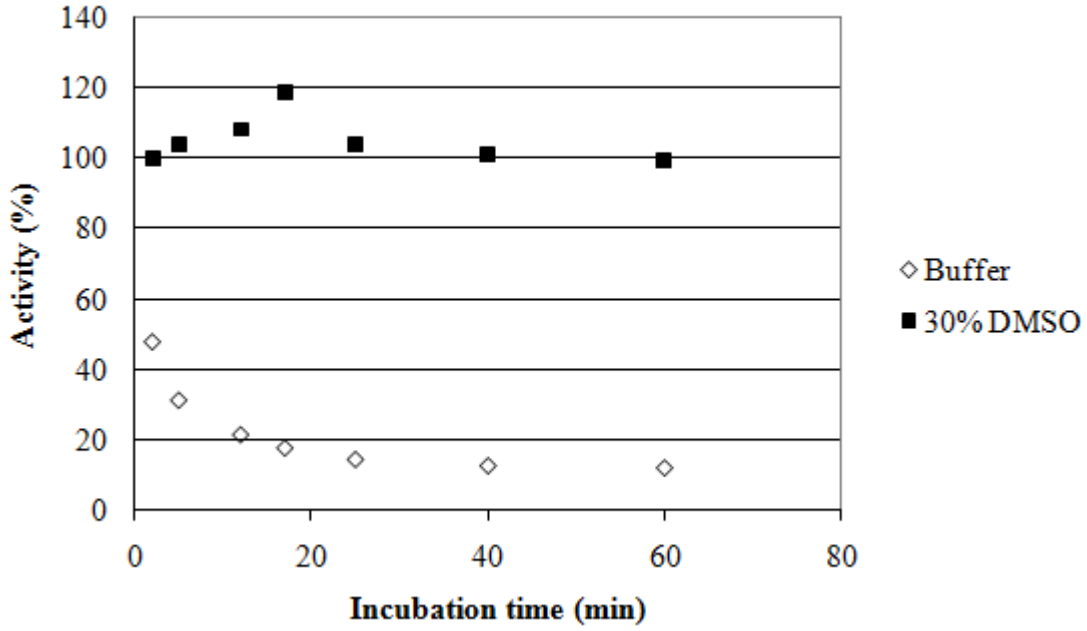


Figure 56: Residual activity of BAL incubated with 0.02 mM ThDP in the presence and absence of 30 vol% DMSO in potassium phosphate buffer at 30°C. Adjusted pH 8.

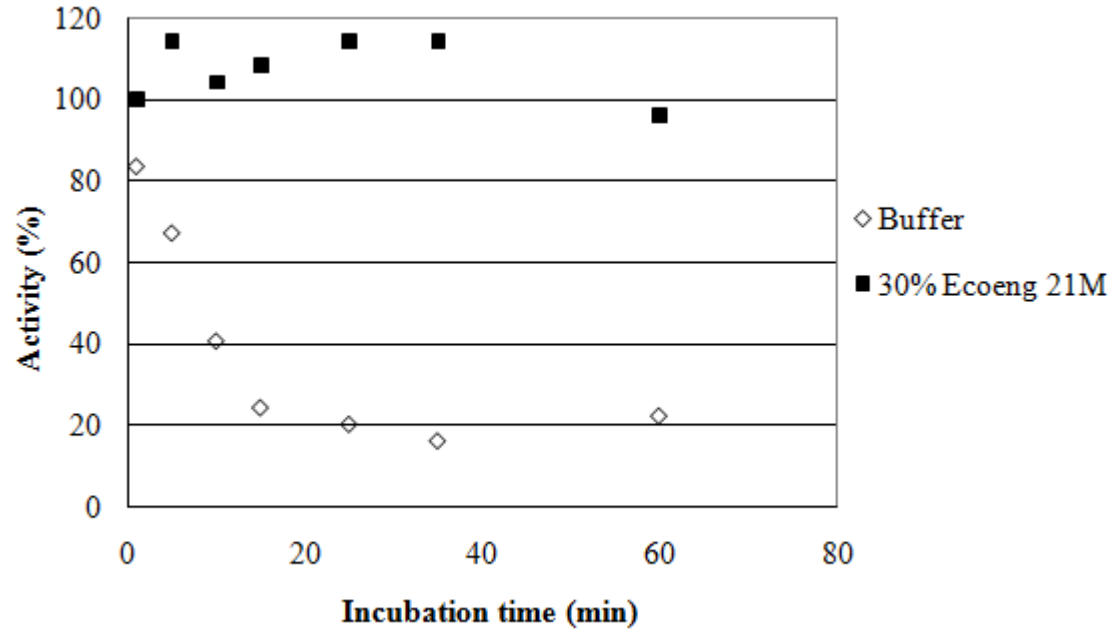


Figure 57: Residual activity of BAL incubated with 0.02 mM ThDP in the presence and absence of 30 vol% Ecoeng 21M in potassium hosphate buffer at 30°C.

Similar studies with BFD*H281A* demonstrated that the enzyme did not lose activity for at least 5 h when cofactors were omitted, regardless of the solvent added. The dissociation of ThDP from BFD was previously also observed to be very slow, with half-life 3 days (**Iding** et al., 2000).

This difference between the ThDP-binding to the polypeptide in both enzyme is a result of structural differences. The ThDP binding motif is highly conserved: in both cases it is realized by the phosphates via Mg^{2+} mediator, coordinated to Asp, Asn and Ser residues as well as to a water molecule. In contrast, structural differences are observed among the amino acids composition around the thiazolium ring of ThDP.

There are two conserved residues in the active sites of both enzymes: alanine (480 in BAL, 460 in BFD) and phenylalanine (484 in BAL and 464 in BFD) (**Hasson** et al. 1998; **Mosbacher** et al. 2005). However, in BAL the chain around the phenylalanine showed a higher mobility (larger *B*-factor) lowering the stability of the enzyme in general. Another interesting observation is the water stripping caused by polyethylene glycole (PEG, which led to shrinkage and compression of Dom γ and′αprobably stabilizing the fixation of ThDP in BAL (**Mosbacher** et al. 2005). These studies indicate that the solvent may have significant impact on the arrangement of the domains, which bind the cofactors. This is probably the reason for the stabilizing effect observed with DMSO and Ecoeng 21M in the case of BAL. In BFD the mobility of the cofactor binding domains is probably lower. Thus, the cofactors are more tightly bound and solvent effects are far less pronounced.

III.4. Enantioselectivity. Effect of cosolvents

The goal of these experiments was not simply to determine the effect of cosolvents on the enantioselectivity of the enzymes but also to use the obtained data to explain other phenomena occurring in non-conventional media. The change of enantioselectivity upon addition of cosolvents will be an indirect proof for structural changes, namely changes in the active site geometry. Two reactions, the synthesis of benzoin from benzaldehyde and the synthesis of 2-hydroxy propiophenone (2-HPP) from benzaldehyde and acetaldehyde have been studied.

III.4.1. Benzoin synthesis

The enantioselectivity of both enzymes in various water/water-miscible solvent mixtures was investigated after 5 hours of reaction. In all cases, the differences in enantiomeric excess were within the range of experimental error (± 2%) (**Table 4**). The presence of organic solvents and ionic liquids did not affect the enzyme enantioselectivity,

implying that the active site shape does not change or the possibly changes are too small to influence the enantioselectivity of this reaction.

Table 4: Effect of the cosolvents on *(R)*-benzoin enantioselectivity.
The reaction media (30°C) consisted of cosolvent mixtures with 50 mM potassium phosphate buffer (50 mM, pH 8 for BAL, pH 6.5 for BFD*H281A*), containing 2.5 mM $MgSO_4$, 0.5 mM ThDP, and 20 mM benzaldehyde. Reactions were started by addition of 20 µg/ml BAL or 100 µg/ml BFD. Samples were taken after 24 hours and subjected to chiral separation using HPLC at room temperature. pH adjusted to 8 (BAL) and 6.5 (BFD*H281A*).

Solvent	BAL *ee* (%)	BFD*H281A* *ee* (%)	Solvent	BAL *ee* (%)	BFD*H281A* *ee* (%)
Buffer	99.0	98.5			
10% DMSO	100.0	100.0	10% Acetone	98.9	99.0
20% DMSO	99.0	98.3	20% Acetone	98.5	100.0
30% DMSO	98.6	98.2	30% Acetone	98.2	99.2
40% DMSO	99.5	99.5	40% Acetone	98.0	99.0
50% DMSO	98.3	98.3	50% Acetone	82.3	98.3
10% Ethanol	100.0	99.3.	10% Ecoeng 1111P	98.3	100.0
20% Ethanol	99.2	98.4	20% Ecoeng 1111P	97.8	100.0
30% Ethanol	97.2	98.6	30% Ecoeng 1111P	98.2	99.2
40% Ethanol	98.3	99.0	40% Ecoeng 1111P	98.1	100.0
50% Ethanol	97.4	98.3	50% Ecoeng 1111P	-	99.3
10% Propanol	98.0	n.d.	10% Ecoeng 21M	99.2	98.3
20% Propanol	100.0	n.d.	20% Ecoeng 21M	100.0	96.7
30% Propanol	100.0	n.d.	30% Ecoeng 21M	99.1	99.0
40% Propanol	99.1	n.d.	40% Ecoeng 21M	98.9	98.0
50% Propanol	97.2	n.d.	50% Ecoeng 21M	99.5	99.8

III.4.2. 2-HPP synthesis

The enantioselectivity of the benzoin synthesis was not affected by the solvents, which means that no severe structural changes occur at the active site. However, minor changes will most probably be better detected by following the enantioselectivity of the 2-

HPP synthesis. The latter has the advantage that the stereoselectivity of the carboligation with both enzymes is not as high as for the benzoin formation, which is catalysed with high *ee* by both enzymes. Especially the variant BFD*H281A* catalyses the synthesis of (*S*)-2-HPP with much lower *ee* (35.4 % in 50 mM potassium phosphate buffer containing 2.5 mM ThDP and 0.5 mM ThDP, pH 6.5, 30 °C, 20 mM benzaldehyde, 200 mM acetaldehyde, **Table 5**) compared to wtBFD (92 % under the same conditions) (**Iding** et al., 2000), whereas wtBAL catalyses the formation of (*R*)-2-HPP with *ee* 91.2 %, i.e. the reaction catalysed by BFD including BFD*H281A* is *S*-selective, whereas BAL produces predominantly the *R*-form. In BFD, the acetaldehyde fits to a tiny *S*-pocket (**Knoll** et al., 2006) and if any changes occur they would be more expected in BFD.

In a reaction media containing both benzaldehyde and acetaldehyde, the 2-HPP synthesis occurs in parallel to benzoin synthesis; however, with BAL the benzoin reacts further with acetaldehyde resulting only in 2-HPP as a final product. With BFD*H281A*, both products are present at the end because the enzyme is not able to accept benzoin as a substrate (see **Table 1**).

The chromatogram in **Fig. 58** clearly shows the difference between the reactions catalysed by both enzymes.

As demonstrated in **Table 5**, the cosolvents affect remarkably the enantioselectivities of both enzymes. In all cases the cosolvents had a positive effect on the enantioselectivity of BAL and the *ee* shifted from 91.5 % to 100%. However, the enantioselectivity of BFD*H281A* was influenced positively by ionic liquids and negatively by organic solvents.

The main reason for this difference between the enzymes can be the presence of the *S*-pocket in the active site of BFD*H281A*, which is just large enough to bind acetaldehyde as an acceptor and explains the rare *S*-selectivity of wtBFD in case of 2-HPP, although benzoin is formed *R*-selectively. As no *S*-pocket is present in BAL, this enzyme is *R*-selective independent of the products formed. It can be assumed that minor structural changes may affect the small *S*-pocket more pronounced than other parts of the active site, which in turn will predominantly affect the enantioselectivity of the BFD variant. Therefore any minor change in the molecule can result in different enantioselectivity in reactions involving substrates which fit in the *S*-pocket (i.e. acetaldehyde). Most probably, such changes occur in ionic liquids and facilitate the access of the molecule into the *S*-

pocket, thus increasing the enantioselectivity. The organic solvents make the molecule more compressed and rigid; therefore, entering the *S*-pocket will become more difficult.

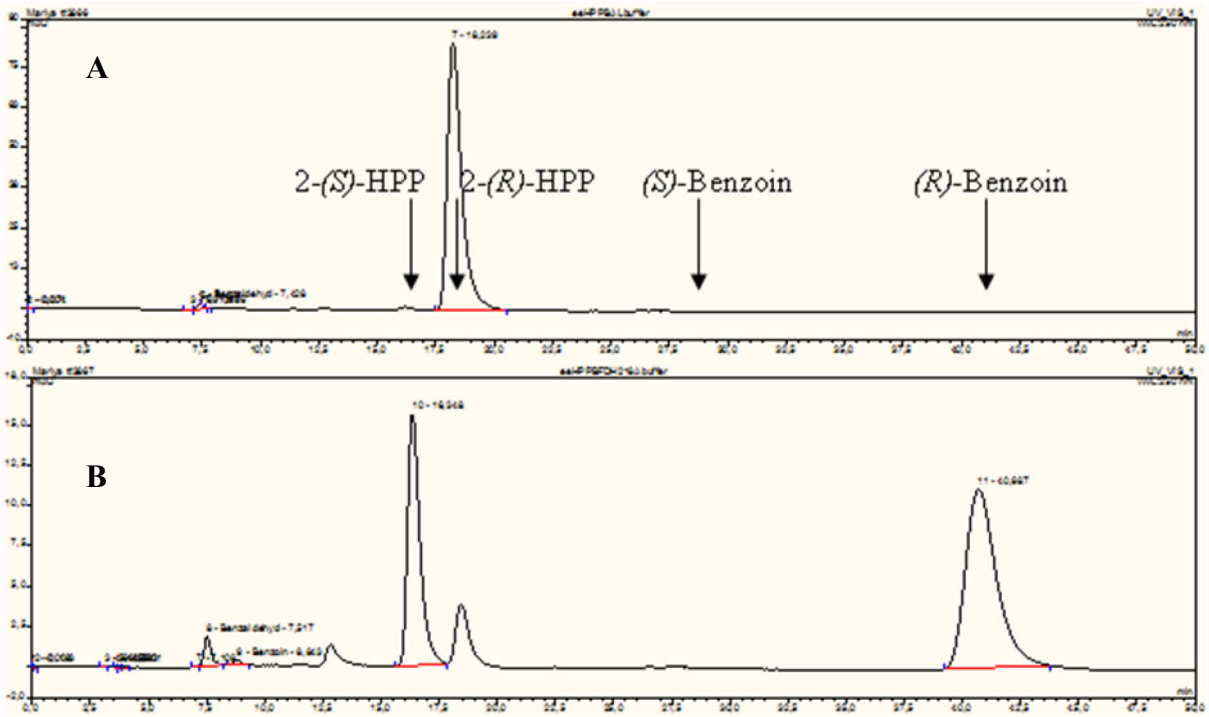


Figure 58: Chromatogram presenting the products of the carboligation reaction of BAL and BFD*H281A*.
The reaction media (30°C) consisted of mixtures of cosolvents with 50 mM potassium phosphate buffer (50 mM, pH 8 for BAL, pH 6.5 for BFD*H281A*), containing 2.5 mM $MgSO_4$, 0.5 mM ThDP, 20 mM benzaldehyde and 200 mM acetaldehyde. Reactions were started by addition of 20 µg/ml BAL or 100 µg/ml BFD. Samples were taken after 24 hours, extracted with hexane:2-propanol (90:10) and separated with a Chiralcel OD-H column from Diacel at room temperature. Retention times: benzaldehyde 12.7 min, 2-*(S)*-HPP 16.1 min, 2-*(R)*-HPP 18.3 min, *(S)*-benzoin 28.5 min, *(R)*-benzoin 40.6 min.

In addition, the changes in enantioselectivity observed with varying concentrations of organic media could be explained by differences in the ***substrate activity***. The substrate activity – in this case activity of benzaldehyde – is defined similarly to the water activity as the availability of the substrate for the catalyst and it depends on its interaction with the solvent. It was previously found that the enantioselectivity of the (*S*)-HPP formation is a function of the benzaldehyde concentration (**Siegert** et al., 2005). It was also shown that the wild-type BFD (**Iding** et al. 2000), as well as several BFD variants (**Siegert** et al., 2005) showed best enantioselectivity with benzaldehyde concentrations below 5 mM. The molecular reason for this unusual behaviour has not yet been elucidated. In this experiment 20 mM benzaldehyde and 200 mM acetaldehyde were used as substrates. The substrate activity rises in the presence of increasing concentrations of cosolvents because its

solubility increases. As a consequence the enantioselectivity in organic solvents could decrease.

Table 5: Effect of solvents on the enantioselectivity of the 2-HPP formation.
The reaction media (30°C) consisted of cosolvents mixtures with 50 mM potassium phosphate buffer (50 mM, pH 8 for BAL, pH 6.5 for BFD*H281A*), containing 2.5 mM $MgSO_4$, 0.5 mM ThDP, 20 mM benzaldehyde and 200 mM acetaldehyde. Reactions were started by addition of 20 µg/ml BAL or 100 µg/ml BFD, respectively.

Solvent	**BAL**		**BFDH*281A***		**Solvent**	**BAL**		**BFDH*281A***	
	(*R*)-HPP		**(*S*)-HPP**			**(*R*)- HPP**		**(*S*)-HPP**	
	conv. (%)	***ee* (%)**	**conv. (%)**	***ee* (%)**		**conv. (%)**	***ee* (%)**	**conv. (%)**	***ee* (%)**
Buffer	100	91.2	81.5	35.4					
10% DMSO	100	87.5	97.5	33.8	10% Acetone	99.3	90.7	84.0	27.2
20% DMSO	100	90.4	95.5	34.7	20% Acetone	94.2	92.6	56.0	24.7
30% DMSO	46.0	100	80.0	35.6	30% Acetone	98.3	93.5	35.0	14.0
40% DMSO	90.0	100	65.0	32.8	40% Acetone	<1	100	20.0	12.8
50% DMSO	91.4	100	35.0	31.7	50% Acetone	2	100	6.0	8.4
10% Ethanol	100	90.1	84.5	29.8	10% Ecoeng 1111P	98.4	88.5	96.5	44.6
20% Ethanol	100	92.4	65.0	24.9	20% Ecoeng 1111P	97.7	90.8	92.5	50.6
30% Ethanol	98.3	100	37.5	17.8	30% Ecoeng 1111P	100	88.4	85.5	59.3
40% Ethanol	<1	100	36.0	14.7	40% Ecoeng 1111P	94.5	94.0	85.5	62.6
50% Ethanol	2	100	31.0	55.5	50% Ecoeng 1111P	96.1	95.0	68.0	62.0
10% Propanol	100	90.7	n.d.	33.5	10% Ecoeng 21M	100	88.0	75.0	35.7
20% Propanol	100	97.2	n.d.	21.9	20% Ecoeng 21M	100	85.1	82.5	37.9
30% Propanol	76.6	100	n.d.	8.7	30% Ecoeng 21M	97.1	92.5	70.5	39.6
40% Propanol	<1	100	n.d.	10.6	40% Ecoeng 21M	98.0	92.4	56.5	41.5
50% Propanol	<1	100		-	50% Ecoeng 21M	100	100	24.5	34.3

III.5. Kinetics and reaction mechanism of benzoin synthesis in selected water/organic solvent mixtures

The complete understanding of the influence of organic solvents on the enzyme activity (**Chapter III.1.**) requires a detailed study on the reaction kinetics and mechanism. To determine the effect of organic solvents on both enzymes, the following reaction systems were used:

1. BAL in aqueous buffer without cosolvent.
2. BAL in aqueous buffer +10 vol% DMSO.
3. BAL in aqueous buffer + 20 vol% acetone.
4. BFD*H281A* in aqueous buffer without cosolvent.
5. BFD*H281A* in aqueous buffer + 10 vol% DMSO.
6. BFD*H281A* in aqueous buffer + 20 vol% DMSO.
7. BFD*H281A* in aqueous buffer + 20 vol% acetone.

There are several reasons to select these particular systems. Two different solvents had to be chosen to distinguish between phenomena due to the solvent and more general phenomena. DMSO was interesting because it improves the reaction rates of both enzymes at lower concentrations; therefore the effect of 10 vol% DMSO was tested on both enzymes. The effect of 20 vol% DMSO was tested only on BFD*H281A* as the rates of BAL in the presence of 10 and 20 vol% DMSO were very similar (**Fig. 8**); therefore the latter system was omitted. Acetone, the second solvent, was added at higher concentration (20 vol%) to test conditions under which the enzymes are significantly inactivated (**Fig. 23**). Finally, all these systems had to be compared with aqueous buffer without additional cosolvent.

The seven selected systems were investigated using macro kinetic studies by initial rate measurements, micro kinetics by progress curve analysis and ^{1}H NMR spectroscopy for analysis of reaction intermediates. As a model reaction, the benzoin synthesis was chosen.

III.5.1. Macro kinetics

The reaction rate *v* was measured as a function of the substrate concentration [*S*] and the obtained experimental data were fitted according to the Michaelis-Menten equation:

$$v = \frac{V_{\max}[S]}{K_M + [S]} \qquad \textbf{(eq. 12)}$$

In case of BAL, the v_0-[S] plots of the experimental data showed hyperbolic curves approximately up to the solubility limit of the substrate benzaldehyde (**Fig. 59**). At higher benzaldehyde concentrations the enzyme is most probably inhibited by the formation of a second (benzaldehyde) phase, which can explain the decay of activity with higher benzaldehyde concentrations.

To determine the theoretical V_{max} (maximum reaction rate if the substrate was soluble over the whole concentration range) the equation was fitted only to the data below the solubility limit (for solubility of benzaldehyde and benzoin see **Appendix, Table A4**). The addition of DMSO and acetone improves the solubility of benzaldehyde in aqueous buffer, enabling the use of higher benzaldehyde concentrations for biocatalysis. The solubility of benzaldehyde in buffer without cosolvents is ~36 mM at 30°C, whereas addition of 10 vol% DMSO allows complete solution of ~70 mM benzaldehyde.

Moreover, the cosolvents accelerated the reaction rate even at low substrate concentration. The reaction rate of BAL with 20 mM benzaldehyde was 19.7 ± 2.1 s^{-1} (in buffer), 51.4 ± 4.1 s^{-1} (in 10 vol% DMSO) and 23.6 ± 2.2 s^{-1} (in 20 vol% acetone) (**Table 6**).

Interestingly, the v_0-[S] plots of BFD*H281A* showed only a linear increase until ~50 mM benzaldehyde indicating that the maximum reaction rate was not achieved. With 20 mM benzaldehyde, the enzyme is thus by far not saturated (**Fig. 59**). Compared to BAL, BFD*H281A* was less sensitive to inactivation phenomena. At higher concentrations, the enzyme was inhibited only in buffer without addition of cosolvent, which indicated that the inactivation was due to the formation of second phase.

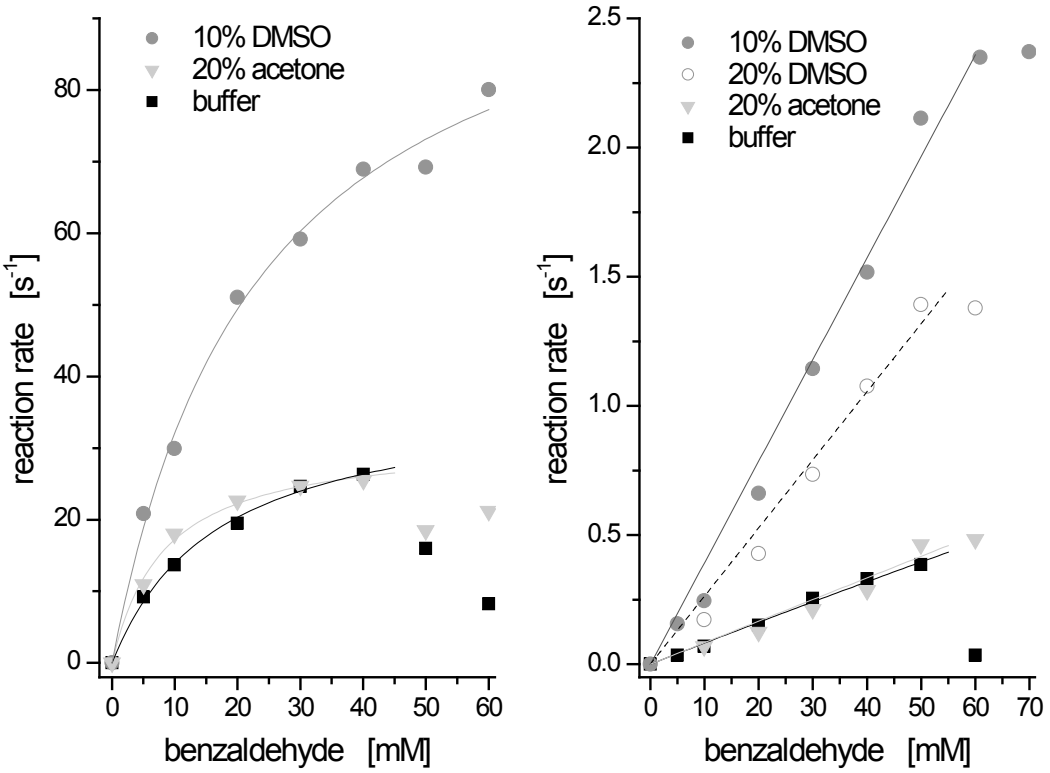


Figure 59: Dependence of the catalytic activity of BAL and BFD*H281A* on the substrate concentration. Solvents were mixed with 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM $MgSO_4$, pH 8 (BAL) and 6.5 (BFD*H281A*), pH was not adjusted after addition of solvent, 30°C.

Table 6: Calculated macrokinetic parameters.
V_{max} and K_M of BFD*H281A* could not be calculated from the direct plots as no hyperbolic kinetics was observed. Details as in **Fig. 59**.

Enzyme	Cosolvent	V_{max} (s^{-1})	K_M (mM)	Reaction rate in 20 mM benzaldehyde* (s^{-1})
BAL	-	58.2±10	16.9±8.0	19.7±2.1
	10 vol% DMSO	110.4±10	26.5±5.2	51.4±4.1
	20 vol% acetone	50.2±8	24.5±10.0	23.6±2.2
BFD*H281A*	-	-	-	0.17±0.03
	10 vol% DMSO	-	-	0.74±0.08
	20 vol% DMSO	-	-	0.48±0.03
	20 vol% acetone	-	-	0.14±0.03

*- concentration used for activity and ^1H NMR studies.

The reaction rate of BFD*H281A* in buffer with 20 mM benzaldehyde was 0.17 ± 0.03 s^{-1}, which is about two orders of magnitude lower than that of BAL. However, the addition of DMSO to the reaction mixture caused a similar effect: it significantly improved the reaction rate to 0.74 ± 0.08 s^{-1} and 0.48 ± 0.03 s^{-1} in the presence of 10 and 20 vol% DMSO, respectively. The reaction rate in 20 vol% acetone was estimated to be 0.14 ± 0.03 s^{-1} (**Table 6**).

III.5.2. Micro kinetics

Following the Michaelis-Menten kinetics only apparent K_M values can be calculated with the assumption that the first and the second benzaldehyde molecules have the same affinity to the active site. However, this assumption is mechanistically not valid, as the binding of the second benzaldehyde molecule will be influenced by the first one. With the help of the mechanistic kinetic model described in **Chapter I.3.4** and **II.5.2**, the two different K_M values (K_{mA} and K_{mB}) can be estimated.

The changes of substrate and product concentrations per time in the seven reaction systems were determined using the HPLC benzoin synthesis assay (**II.4.4.4**). Thereafter, the obtained progress curves were fitted according to the model (**Fig. 60)**.

K_{eq} was determined to be 3.019 mM^{-1}. The obtained parameter estimates together with their standard deviations are listed in **Table 7**. The data demonstrate that the maximum turnover number k_{catf} can be estimated very precisely. The precision for K_{mA} and K_{mB} is also satisfactory. Apparently, the cosolvents affect all parameters, although their strongest impact is on k_{catf}. Both enzymes show higher k_{catf} in presence of the cosolvent compared to buffer with only one exception – BFD*H281A* in 20 vol% acetone. The addition of 10 vol% DMSO caused the strongest effect on both enzymes.

By comparison of the values obtained for both enzymes, it becomes apparent that BAL is about one order of magnitude more active with respect to benzoin synthesis than BFD*H281A*. This is not surprising since BAL is known to be significantly more active concerning benzoin formation (**Demir** et al., 1999). In all cases, the K_{mB}-values are higher than K_{mA} (**Table 7**). Compared to BAL, the K_M values of BFD*H281A* are huge, as described previously for wt BFD and propanal as a substrate (**Mikolajek** et al., 2007). This extremely low affinity for aliphatic and aromatic aldehyde substrates is probably a specific

property of BFD, which explains why V_{max} cannot be reached with this enzyme in aqueous/organic solvent systems (**Fig. 59**).

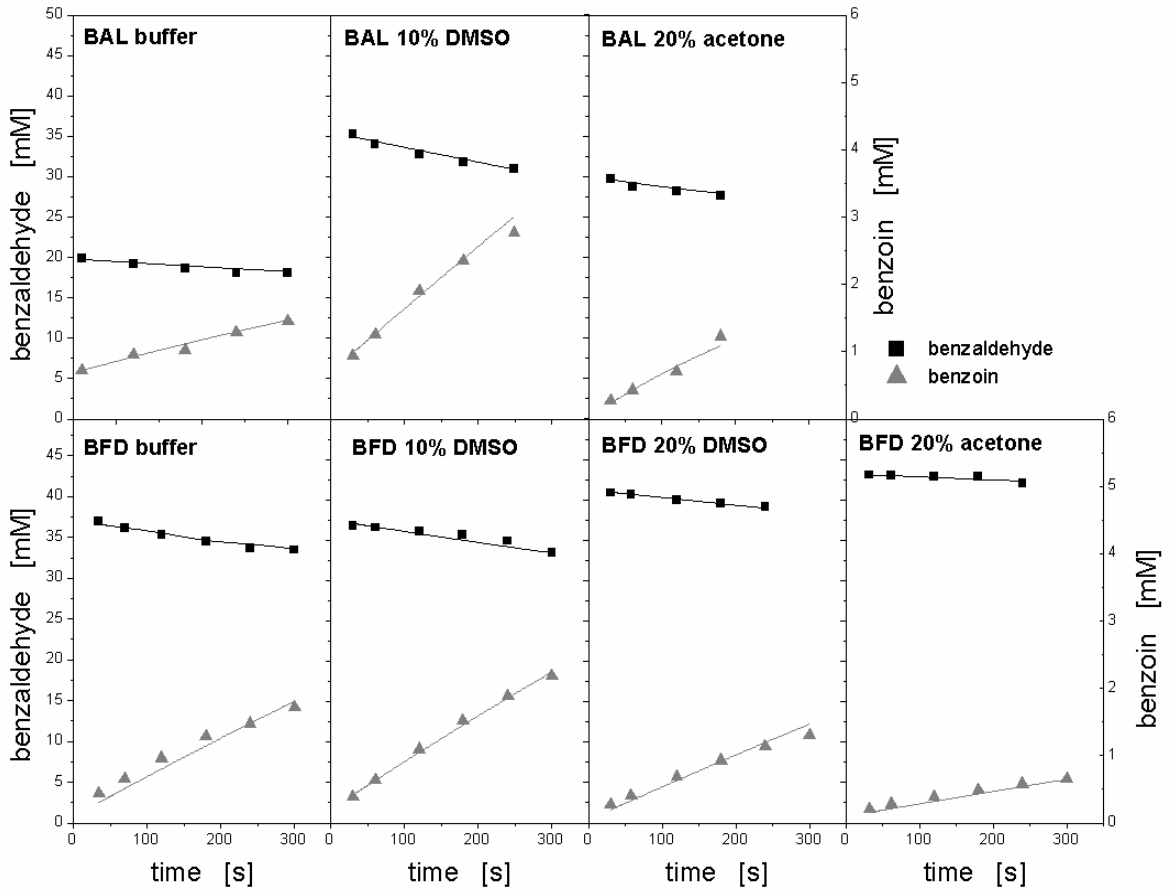


Figure 60: Progress curves fitted by the kinetic model.
The progress curves of substrate and product over time were fitted using **eq. 4**. Solvents were mixed with 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM $MgSO_4$, pH 8 (BAL) and 6.5 (BFD*H281A*) in buffer, 30°C. pH was not adjusted after addition of solvent.

The dependent parameters calculated using **eq. 5-7** are presented in **Table 8**. Obviously, the solvents affected also the inhibitory constants and the Michaelis constant of the product. Once again, DMSO proved to be the better cosolvent (at 10 vol% concentration) as it considerably increases all the parameters. Due to instability of BFD*H281A* in 20 vol% acetone, the parameters differ to some degree but still show the same tendency. The other cases (BAL in 20 vol% DMSO and BFDH*281A* in 20 vol% acetone) showed almost no change compared to the buffer system.

Table 7: Estimated independent parameters for the formation of benzoin from benzaldehyde.
Solvents were mixed with 50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.5 mM $MgSO_4$, pH 8 (BAL) and 6.5 (BFD*H281A*) not adjusted after addition of solvent, with 20 mM benzaldehyde, 30°C.

Enzyme	Organic solvent	K_{eq} (mM^{-1})	K_{catf} (s^{-1})	K_{mA} (mM)	K_{mB} (mM)
BAL	-	3.019	35.1 ± 0.4	0.28 ± 0.05	0.39 ± 0.06
	10 vol% DMSO	3.019	78.1 ± 0.9	1.96 ± 0.55	6.13 ± 0.08
	20 vol% acetone	3.019	39.9 ± 0.6	1.47 ± 0.12	1.47 ± 0.12
BFD*H281A*	-	3.019	3.5 ± 0.5	131.3 ± 23.9	139.5 ± 23.1
	10 vol% DMSO	3.019	11.7 ± 0.6	86.5 ± 8.5	109.5 ± 7.4
	20 vol% DMSO	3.019	16.8 ± 2.6	234.3 ± 42.0	242.4 ± 42.0
	20 vol% acetone	3.019	2.4 ± 0.4	42.5 ± 22.6	82.2 ± 18.8

Table 8: Calculated dependent parameters for the enzymatic benzoin formation.
The dependent parameters were calculated using **eq. 5-7** and the data from **Table 7**.

Enzyme	Organic solvent	*KiA* (mM)	*KmP* (mM)	*KiB* (mM)
BAL	-	0.11	0.02	0.22
	10 vol% DMSO	4.18	82.62	8.36
	20 vol% acetone	0.15	0.04	0.30
BFDH*281A*	-	8.19	107.58	16.38
	10 vol% DMSO	23.00	1011.19	46.01
	20 vol% DMSO	8.17	104.18	16.33
	20 vol% acetone	39.76	4620.28	79.51

The micro-reaction constants (**Fig. 61**) were calculated according to **eq. 8-11**. In this figure the calculated values are not only presented as numbers, but are also visualized in terms of bar charts. Thus, different kinds of kinetic limitations can be clearly seen for

BAL and BFD*H281A*. Catalysis of BAL is mostly rate-determined by product release (see **Fig. 3**, *step 3*). Moreover, the bar charts clearly visualize the kinetic effect of the cosolvents: they accelerate the release of benzoin, while they decrease the substrate binding rate. However, the overall reaction rate is increased since the product release is the bottleneck of the reaction. Taking into account that k_1 and k_2 are second order rate constants, it may be concluded that above 4 mM substrate concentration the product release is rate-limiting. The same situation holds true in 10 vol% DMSO. These results are in line with recently reported data. For BAL-catalyzed synthesis of *(R)*-3,3',5,5'-tetramethoxybenzoin this was detected by using a mechanistic kinetic model (**Zavrel** et al., 2008). Moreover, CD spectroscopic data indicated that the release of benzoin could be rate-limiting for BAL-catalyzed benzoin synthesis (**Chakraborty** et al., 2008).

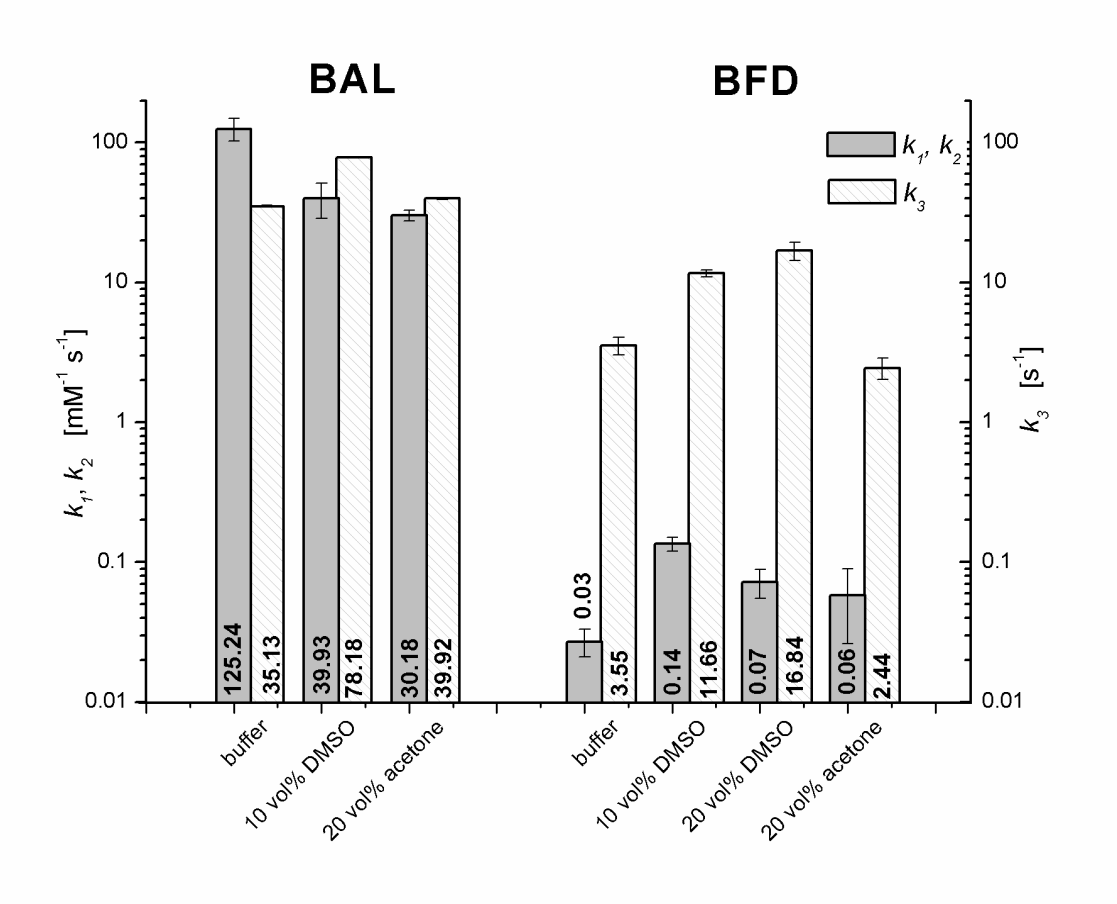


Figure 61: Micro-reaction constants of benzoin formation.
The dependent parameters were calculated using **eq. 8-11** and the data from **Table 7**. The constants k_1 and k_2 are identical, k_3 equals k_{catf}. The product formation is rate limiting with BAL, and the fastest reaction with BFD*H281A*. Cosolvents improve the rates of limiting steps.

In contrast, the product release is the fastest reaction step for the benzoin formation catalyzed by BFD*H281A*, whereas the carboligation (**Fig. 3**, *step 2*), was identified as the rate-limiting step. The results clearly demonstrate that DMSO is a better cosolvent than acetone, as all micro-reaction constants were increased in DMSO but decreased in acetone.

The different rate limitations with BAL and BFD*H281A* can be rationalized in terms of different active site geometry. As already discussed, structural studies show that the active site of BAL is partly covered by a C-terminal helix (**Fig. 4**, **5**), which could hinder product release. In BFD, such a structural element is absent, which might allow faster product release. Moreover, the active site pocket of BFD is smaller than that of BAL and the benzoin molecule could experience steric stress (**Knoll** et al., 2006), which would enforce it to leave the active site immediately after its formation.

III.5.3. ^{1}H NMR spectroscopy

To test the reliability of the progress curve analysis, a steady-state intermediate analysis employing ^{1}H NMR spectroscopy as an analytic tool was carried out. The ^{1}H NMR spectroscopy gives the unique possibility to directly observe the key intermediates of benzoin synthesis. Using the established method detailed in (**Tittmann** et al., 2003) the relative distribution of reaction intermediates ThDP, hydroxybenzyl-ThDP (HBz-ThDP) and benzoin-ThDP (**Fig. 3**) in steady-state can be determined. Using these data individual net rate constants can be calculated on the basis of the proton signal integrals of ThDP and of derived intermediates.

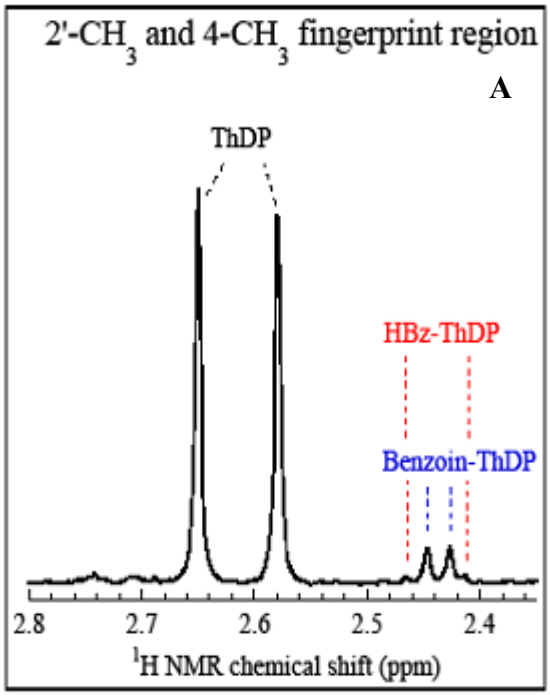
However, in the current study, these results are treated as qualitative for two main reasons: first of all, the v_0-[S] plot (**Fig. 59**) demonstrated that owing to limited solubility of the substrate V_{max} was not achieved under the deployed reaction conditions (20 mM benzaldehyde). Secondly, it was observed that BAL rapidly lost the cofactor ThDP from the active sites when dissolved in potassium phosphate buffer without excess cofactors (**III.3.3**). Unfortunately, the ^{1}H NMR analysis cannot distinguish between free and enzyme-bound ThDP; thus making the quantitative estimation of enzyme-bound C2-unsubstituted ThDP not reliable.

When BAL was reacted with benzaldehyde without any cosolvent, all intermediates (ThDP, HBz-ThDP, benzoin-ThDP) were identifiable in the NMR spectra (**Fig. 62A**). The

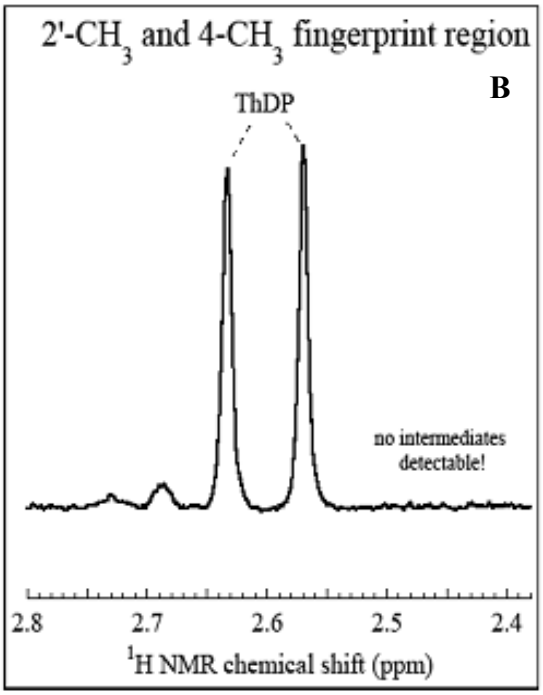
amount of benzoin-ThDP was higher than that of HBz-ThDP, confirming that the release of benzoin is the rate-limiting unimolecular step. In the presence of 20 vol% acetone, only C2-unsubstituted ThDP was detectable indicating that the product release is accelerated as already suggested by the progress curve analysis (**Fig. 62B**).

Unlike BAL, BFD*H281A* did not lose ThDP in buffer without excess cofactors. In this case, the high concentrations of C2-unsubstituted ThDP quantified by NMR were caused only by non-saturation of the enzyme as a consequence of the extremely high K_M values as discussed above. The v_0-[S] plot (**Fig. 59**) shows an almost linear increase up to the solubility limit and no hyperbolic dependence is observable; therefore, the active sites were by far not saturated under the chosen reaction conditions (20 mM benzaldehyde).

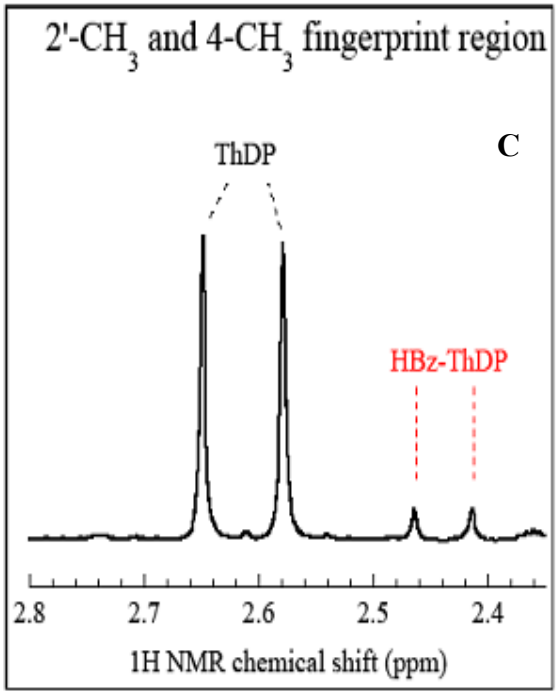
Quantitative ^{1}H NMR analysis of intermediates formed in the course of BFD*H281A*-catalyzed synthesis of benzoin revealed that in all cases only ThDP and HBz-ThDP were accumulated at the steady-state (**Fig. 62 C+D**), clearly indicating that carboligation (that is the addition of the second molecule benzaldehyde to HBz-ThDP) is rate-determining for the overall reaction. Addition of a cosolvent (10, 20 vol% DMSO or 20 vol% acetone) slightly accelerates carboligation relative to HBz-ThDP formation but still the carboligation is rate-limiting. The product release is the fastest elementary reaction step as no benzoin-ThDP adduct could be observed, so once the benzoin-ThDP molecules are being formed, the product will immediately split off from ThDP. As stated before, these results are in good agreement with the results obtained by the progress curve analysis.



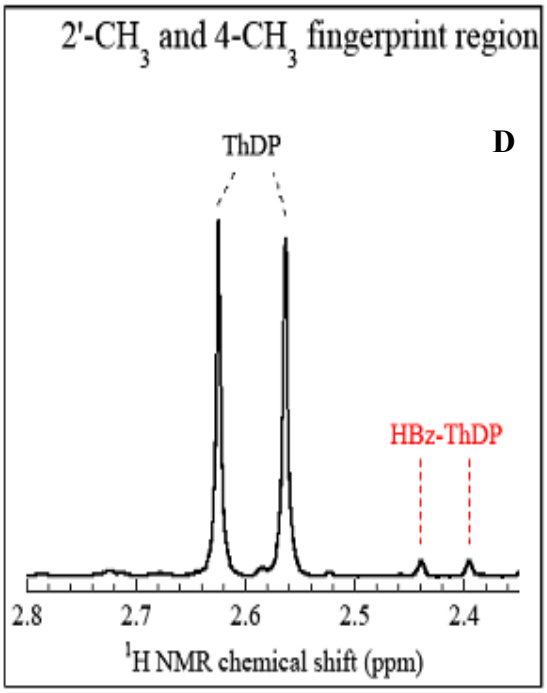
(A) - BAL in aqueous buffer



(B) - BAL in 20% acetone



(C) – BFD*H281A* in aqueous buffer



(D) – BFD*H281A* in 20% acetone

Figure 62: Distribution of reaction intermediates of enzyme catalysed benzoin formation.
BAL (6 mg/ml) or BFD*H281A* (10 mg/ml) were reacted in cofactor-free solvents, 30°C, 20 mM benzaldehyde, for 1-2 s to assure steady-state conditions and stopped by addition of 12.5 (w/v) TCA/1 M HCl (in D_2O). Proteins were precipitated and discarded, supernatant subjected to 1D ^{1}H NMR spectroscopy at 298 K and using water presaturation techniques. Chemical shifts of 2′-CH_3 and 4-CH_3 ^{1}H NMR singlet signals: ThDP (2.65 and 2.58 ppm), HBz-ThDP (2.47 and 2.42 ppm) and benzoin-ThDP (2.45 and 2.43 ppm).

IV.1. SUMMARY

This thesis reports an extensive investigation of the performance of two structurally similar thiamine-diphosphate dependent enzymes, BAL and BFD*H281A*, in various non-conventional media. First, the effect of cosolvents on the activity and stability was tested. Thereafter, a series of experiment was conducted to determine the molecular reasons for the observed effects. This series included studies on the enzyme solubility, pH effects and pH-shift, cofactor requirement, enantioselectivity using two different reactions, structural studies by fluorescence and circular dichroism spectrometry and investigation of the reaction mechanism using progress curve analysis, kinetic modelling and analysis of reaction intermediates by ^{1}H NMR spectroscopy.

Enzyme activity

Addition of small amounts of organic solvents (except for DMSO) deteriorated the initial reaction rates. In contrast, addition of 10 to 30 vol% DMSO improved the reaction rate of BAL weakly, whereas higher concentrations inactivated the enzyme. The small increase of activity in the presence of 10-20 vol% DMSO was due to an increase of pH. When the pH was kept constant, the enzyme was gradually inactivated in increasing concentrations of DMSO. 10 vol% DMSO had a strong impact on BFDH*281A* causing almost two-fold increase of the reaction rate. When the pH was kept constant, the results were similar to those with increasing pH. This interesting pH effect was studied in more detail with BFD*H281A*. It was found that in increasing concentrations of DMSO the pH-optimum shifts to the alkaline direction.

In ionic liquids, the activity of BAL was considerably improved in presence of low concentrations of Ecoeng 21M and Ecoeng 1111P. The ionic liquids, being salts, caused a salting-out effect on the enzymes which was shown to be a major reason for their inactivation. Small amounts of DMSO and ionic liquids probably induce more active enzyme conformations which might be due to their function as molecular lubricants.

As expected, the reaction rate in water-free environment was much lower than in aqueous medium. In pure water-immiscible organic solvents BAL was 200,000 times less active than in aqueous buffer and completely inactive in $scCO_2$. BFD*H281A* produced only traces of benzoin under supercritical conditions, which can be explained by its higher

thermostability, which allows the enzyme to work longer. Several reasons could contribute to this lack of activity in water-free environment:

- The enzymes were not soluble in water-free solvents.
- The cofactors were not soluble in water-free solvent or not sufficient.
- The active site and cofactor binding site change their structure.
- The water content and/or water activity were too low to allow catalysis.

Enzyme stability

The effects of solvents on both enzymes were different. The stability of BAL was better in presence of DMSO than in aqueous buffer, whereas the stability of BFD*H281A* was best in buffer. Ecoeng 21M had a slightly positive effect on BAL but caused destabilisation of BFD*H281A*. However, water-miscible solvents and ionic liquids deactivated both enzymes immediately and irreversibly. In general, the best conditions for stability were those at which the enzymes were most active, and *vice versa*.

The phenomenon of increased BAL stability in 30 vol% DMSO compared to buffer was analysed in more detail. It was shown that this stabilisation was not due to suppressed bacterial growth. Instead, it can be discussed as an effect of the lowered water activity, which makes the enzyme structure more rigid (water is involved in the enzyme inactivation). Moreover, it can be explained by the improved ThDP-binding to the active site in presence of DMSO.

Table 9 summarizes and compares the stability of BAL and BFD*H281A* in different media at 30°C.

Cofactor stability and dissociation

The ThDP binds to the active site by non-covalent interactions. Therefore, it was expected that the addition of cosolvents will affect the cofactor association. It was demonstrated that BAL activity in aqueous buffer without cofactors drops rapidly due to cofactor loss. Interestingly, in 30 vol% DMSO or Ecoeng 21M without added cofactors the enzyme activity remained unchanged, proving that the cosolvents stabilize the cofactor binding to the active site.

In contrast, BFD*H281A* kept the cofactor under all conditions tested, which is an indication for stronger ThDP-binding to the active site.

Table 9: BAL and BFD*H281A* stability in different media - summary.

Solvent	Half-life of BAL (pH 8)	Half-life of BFD*H281A* (pH
Buffer*	32-81 h	>2 weeks
DMSO	98.4 h (30 vol% DMSO)	>2 weeks (10, 20 vol% DMSO)
20 vol% Ecoeng 21M	137 h	100 h
100% Petroleum ether	12.6 h	n.d.
100% MTBE,	2.2 h	n.d.
100% organic solvents (acetone, ethanol, 2-propanol, DMSO) and ionic liquids	<1 min	<1 min
Supercritical CO_2	<1	173

*- 50 mM potassium phosphate buffer, 0.5 mM ThDP, 2.5 mM $MgSO_4$

Enzyme structure

The fluorescence analysis showed similar trends with both enzymes. The ionic liquids caused red shift of emission maximum which means unfolding, whereas the organic solvents caused blue shift which was interpreted as loss of hydration water and shrinking of the molecule. This experiment proved that the enzymes followed different inactivation mechanisms in organic solvents and in ionic liquids.

The CD analysis demonstrated that the organic solvents acetone and ethanol did not unfold the enzymes confirming the results from tryptophan fluorescence studies. Unfortunately, it was impossible to measure the effect of DMSO and ILs because they interfere with the signal from the α-helix.

Enantioselectivity

The enantioselectivity of the benzoin formation was not affected by organic solvents and ionic liquids. However, the enantioselectivity of the 2-HPP synthesis from acetaldehyde and benzaldehyde showed a very interesting difference. With BAL, it increased upon addition of cosolvents. With BFD*H281A*, the ionic liquids improved the enantioselectivity, whereas the organic solvents deteriorated it. This difference was interpreted as a minor structural change in the *S*-pocket of active site of the BFD variant, which can affect the position of acetaldehyde prior to C-C bond formation in the active site.

Reaction kinetics and mechanism

The studies on the reaction kinetics and mechanism showed that the presence of DMSO and acetone affected the reaction parameters, the micro-reaction constants and the distribution of reaction intermediates. The initial reaction rate analysis confirmed the positive effect of cosolvents of the reaction rate. They did not only accelerate the reaction but also improved the substrate solubility; thus allowing higher substrate concentrations in the reaction mixture. Moreover, the results showed an interesting feature of BFD*H281A*: the linear increase of the reaction rate up to the solubility limit.

Using a mechanistic kinetic model it was possible to determine the micro-reaction constants and the rate-limiting steps of benzoin condensation catalyzed by BAL and BFD*H281A*. For BAL, the rate-limiting step was the product (benzoin) release; whereas for BFD*H281A*, the rate-limiting step was identified to be the carboligation of HBz-ThDP and benzaldehyde. For BAL, these results were consistent with previous studies (**Zavrel** et al., 2008; **Chakraborty** et al., 2008). To my knowledge, this is the first report succeeding in the identification of rate-limiting steps for carboligation catalyzed by a BFD variant.

IV.2. ZUSAMMENFASSUNG

Gegenstand dieser Doktorarbeit ist die Charakterisierung zweier Thiamindiphosphat (ThDP)-abhängiger Enzymen, Benzaldehydlyase (BAL) und einer Variante der Benzoylformiatdecarboxylase (BFD*H281A*), in verschiedenen unkonventionellen Medien. Dabei stand die Ermittlung der molekularen Ursachen für die Effekte unkonventioneller Medien auf die Aktivität und Stabilität im Mittelpunkt. Hierzu wurden die Einflüsse insbesondere von wasser-mischbaren organischen Lösungsmitteln und ionischen Flüssigkeiten auf die Enzymlöslichkeit, den Kofaktorbedarf und die Enantioselektivität untersucht. Darüberhinaus spielen pH-Effekte eine wichtige Rolle, da es durch den Lösungsmittlezusatz zu pH-Verschiebungen im wässrigen Puffer kommt. Strukturelle Effekte wurden mittels Fluoreszenz- und Circulardichroismus Spektrometrie untersucht. Eine besondere Bedeutung kommen Untersuchung des Reaktionsmechanismus mittels Anfangsreaktionsgeschwindigkeitsbestimmungen, Umsatz/Zeit-Kurven Analyse sowie der Analyse der Reaktionsintermediaten durch ^{1}H NMR Spektroskopie zu.

Enzymaktivität

Grundsätzlich kann festgestellt werden, dass wassermischbare organische Lösungsmittel bereits in geringen Mengen die Aktivität der Enzyme beeinträchtigen. DMSO bildet hier eine Ausnahme. Der Zusatz von 10 bis 30 vol% DMSO verbesserte die Reaktionsrate der BAL schwach, während höhere Konzentrationen das Enzym inaktivierten. Die geringe Zunahme der Aktivität in 10-30 vol% DMSO beruht auf einer Erhöhung des apparenten pH-Werts. Der Effekt verschwindet, wenn der pH konstant gehalten wird., In diesem Fall wurde das Enzym in ansteigenden Konzentrationen von DMSO graduell inaktiviert.

Im Gegensatz dazu bewirken 10 vol% DMSO eine Verdopplung der Aktivität der BFD*H281A*. In diesem Fall verschindet der aktivitätssteigernde Effekt nicht, wenn der pH konstant gehalten wird. Dies liegt daran, dass sich das pH-Optimum der BFD*H281A* katalysierten Benzoinsynthese durch Zusatz von DMSO kontinuierlich ins Alkalische verschiebt.

In Gegenwart niedriger Konzentrationen der ionischen Flüssigkeiten Ecoeng 21M und Ecoeng 1111P wurde die Aktivität der BAL deutlich verbessert. Die ionischen Flüssigkeiten, die Salze sind, verursachen bei höheren Konzentrationen einen Aussalzeffekt auf die Enzyme, was bereits als hauptsächliche Ursache für die Deaktivierung hervorgehoben wurde. Wahrscheinlich induzieren kleine Mengen von DMSO und ionischen Flüssigkeiten aktivere Enzymkonformationen bzw. wirken wie „molekulare Schmiermittel".

Wie erwartet ist die Reaktionsrate in wasserfreien Lösungsmitteln viel niedriger als im wässrigen Puffer. In reinen nicht wassermischbaren organischen Lösungsmitteln ist die BAL 200.000-mal weniger aktiv als im wässrigen Puffer und das Enzym ist völlig inaktiv in $scCO_2$. Dass die BFD*H281A* in unter überkritischen Bedingungen nur Spuren des Benzoins produzierte, kann durch ihre höhere Thermostabilität erklärt werden, die das Enzym bei 40°C länger aktiv sein lässt. Folgende Gründe könnten zu der geringen Aktivität in wasserfreien Medien beitragen:

- Die Enzyme sind nicht in den wasserfreien Lösungsmitteln löslich.
- Die Kofaktoren sind im wasserfreien Lösungsmittel entweder nicht löslich oder nicht ausreichend, um die aktiven Zentren zu sättigen.
- Das aktive Zentrum und die Kofaktorbindestelle ändern ihre Struktur.
- Der Wassergehalt und/oder die Wasseraktivität sind zu niedrig, um Enzymkatalyse zu ermöglichen.

Enzymstabilität

Die Effekte der Lösungsmittel auf beide Enzyme sind unterschiedlich. Die Stabilität der BAL wird durch Zugabe von DMSO im Vergleich zum Puffer verbessert, während die Stabilität der BFD*H281A* im Puffer am besten war. Ecoeng 21M hatte einen geringfügigen positiven Effekt auf die BAL, verursachte aber eine Destabilisierung der BFD*H281A*. Jedoch deaktivieren pure wassermischbare organische Lösungsmittel und ionische Flüssigkeiten beide Enzyme sofort und irreversibel. Im Allgemeinen waren die besten Bedingungen für Stabilität diejenigen, bei denen die Enzyme am aktivsten waren, und umgekehrt.

Das Phänomen der signifikanten Stabilitätsverbesserung der BAL in Gegenwart von 30 vol% DMSO im Vergleich zu Phosphatpuffer wurde detailliert untersucht. Es

konnte gezeigt werden, dass der Effekt nichts mit unterdrücktem bakteriellem Wachstum zu tun hat. DMSO erhöht die strukturelle Rigidität des Enzyms.

Tabelle 9 fasst die Effekte zusammen und vergleicht die Stabilitäten der BAL und der BFD*H281A* in den verschiedenen Medien bei 30°C.

Table 9: Stabilitäten von BAL and BFD*H281A* in verschiedenen unkonventionellen Medien - Zusammenfassung.

Medium	**Halbwertszeit BAL (pH 8)**	**Halbwertszeit BFD*H281A* (pH 6.5)**
Puffer*	32-81 h	>2 Wochen
DMSO	98.4 h (30 vol% DMSO)	>2 Wochen (10, 20 vol% DMSO)
20 vol% Ecoeng 21M	137 h	100 h
100% Petrolether	12.6 h	n.b.
100% MTBE, Hexadecan	2.2 h	n.b.
100% organische Lösungs-mittel (Aceton, Ethanol, 2-Propanol, DMSO) und ILs	<1 min	<1 min
$ScCO_2$	<1	173

*- 50 mM Kaliumphosphatpuffer, 0.5 mM ThDP, 2.5 mM $MgSO_4$

Cofaktorstabilität und -dissoziation

ThDP und Magnesiumionen binden nicht kovalent an die Enzyme. In wässrigen Puffern müssen daher zum Erhalt der Stabilität insbesondere bei der BAL überschüssige Kofaktoren zugesetzt werden. Da unkonventionelle Medien die Struktur der Enzyme beeinflussen können, kann auch ein Einfluss auf die Stabilität der Cofaktorbindung erwartet werden. Es konnte gezeigt werden, dass sowohl DMSO als auch Ecoeng 21M (je 30 vol%) die Stabilität der Cofaktorbindung in der BAL verbessern, so dass das Enyzme auch ohne Zusatz überschüssiger Cofaktoren stabil bleibt.

Demgegenüber konnten bei der BFD*H281A*, die auch unter wässrigen Bedingungen eine stabile Cofaktorbindung zeigt, keine Effekte der unkonventionellen Medien auf die Kofaktorbindung beobachtet werden.

Enzymstruktur

Untersuchungen struktureller Veränderungen der Enzyme mittels Tryptophan-fluoreszenz zeigten bei BAL und BFD*H281A* ähnliche Tendenzen. Während ionische Flüssigkeiten eine Rotverschiebung des Emissionmaximums verursachten, das auf (partielle) Auffaltung bedeutet, bewirken organische Lösungsmittel eine Blauverschiebung, die als Verlust des Hydratationwassers und Schrumpfung des Moleküls gedeutet werden kann. Dieses Experiment bestätigte, dass die Enzyme verschiedenen Inaktivierungmechanismen in organischen Lösungsmitteln und in ionischen Flüssigkeiten unterliegen.

Die Analyse mittels Circulardichoismus bestätigte die Resultate der Tryptophan-Fluoreszenz, dass die organischen Lösungsmittel Aceton und Ethanol die Enzyme partiell entfalten. Leider war es unmöglich, den Effekt von DMSO und von ionischen Flüssigkeiten zu untersuchen, weil deren Absorption im UV-Bereich mit dem Signal der α-Helix interferiert.

Enantioselektivität

Die Enantioselektivität der Benzoinsynthese wurde durch organische Lösungsmittel und ionische Flüssigkeiten nicht beeinflußt. Jedoch wirkten sich die Lösungsmittelzusätze auch die Enantioselektivität der 2-HPP-Synthese aus. Mit der BAL als Katalysator erhöhte sie sich der ee für (*R*)-2-HPP nach Einführung von Lösungsmitteln. Mit der BFDH*281A* verbesserten die ionischen Flüssigkeiten die Enantioselektivität von (*S*)-2-HPP, während die organischen Lösungsmittel sie verschlechterten. Dieser Unterschied wurde als eine kleine Strukturveränderung in der *S*-Tasche des aktiven Zentrums interpretiert, die die Bindung des Akzeptoraldehyds Acetaldehyd beeinflusst.

Reaktionskinetik und Mechanismus

Die Untersuchungen der Reaktionskinetik und des Reaktionsmechanismus zeigten, dass die Gegenwart von DMSO und Aceton die kinetischen Parameter, die Mikroreaktions-konstanten und die Verteilung der Reaktionsintermediaten beeinflußt. Die Untersuchung der Anfangsreaktionsgeschwindigkeiten bestätigte den positiven Effekt der

Lösungsmittel auf die Reaktionsrate. Sie beschleunigen nicht nur die Reaktion, sondern verbesserten auch die Substratlöslichkeit und erlaubten somit höhere Substratkonzentrationen im Reaktionsansatz. Außerdem zeigten die Resultate eine interessante Eigenschaft der BFD*H281A*: die lineare Zunahme der Reaktionsrate bis zur Löslichkeitgrenze des Benzaldehydes, während bei der BAL eine klassische Sättigungskinetik gefunden wurde.

Unter Verwendung eines mechanistischen kinetischen Modells war es möglich, die Mikroreaktionskonstanten und die geschwindigkeitsbestimmenden Schritte der Benzoin-kondensation – katalysiert durch die BAL und die BFD*H281A* – festzustellen. Für die BAL war der geschwindigkeitsbestimmenden Schritt die Freisetzung des Produkts (Benzoin), während für die BFD*H281A* die Carboligation von HBz-ThDP und Benzaldehyd als geschwindigkeitsbestimmend identifiziert wurde. Für die BAL waren diese Resultate mit vorhergehenden Studien in Einklang (**Zavrel** et al., 2008; **Chakraborty** et al., 2008). Meines Wissens nach, wurde in dieser Arbeit erstmal geschwindigkeitsbestimmende Schritte der durch eine BFD Variante katalysierte Carboligation bestimmt.

V. CONCLUSIONS

To really take advantage of non-conventional media, it is important to understand the basic principles and characteristics of these systems. The effect of cosolvents on enzymes is complex. They can destabilize the structure, the binding of the cofactor and they may influence the reaction mechanism, either by influencing the reaction rates of single steps or by inducing changes in pH and water activity.

As research objects two structurally similar enzymes were used: BAL and BFD*H281A*. Both use the same cofactors, are tetramers and catalyse the same reaction. These enzymes provided an excellent objective to study effects of non-conventional media, as they differ in stability and enantioselectivity. Furthermore, the whole catalytic cycle which involves different intermediates covalently bound to thiamine diphosphate is known. As the cofactor is bound non-covalently in a similar mode in both enzymes and can be easily released from the enzyme by acid quenching, it is possible to analyse the micro-reaction constants of the whole catalytic cycle. This technique allows the analysis of influences on the micro-reaction constants by non-conventional media, which is a unique opportunity in enzymology.

By addition of organic solvent into the reaction systems many parameters change depending on the solvent nature: the polarity and ionic interaction, free energy and partition coefficients of all components (**Ducret** et al., 1998). None of these parameters alone can explain the obtained results (change of activity, stability, enantioselectivity). Other factors, such as viscosity (in ILs, **van Rantwjik** and **Sheldon**, 2007), denaturation capacity and toxicity of the solvents, also contribute to the final results. Overall, the effects of organic solvents were comparable except for DMSO, which is apparently most appropriate for the used enzymes.

In addition to the specific parameters, the solvents influence the enzymes indirectly e.g. by changing the structure of water around the enzymes and thus affecting their configuration. The decreased water activity around the enzyme molecule reduces its flexibility and often improves its stability. Stability of BAL in 30°C and 40°C, for example, was improved by addition of 30 vol% DMSO. This stabilizing effect is due to a more stable cofactor binding in the presence of DMSO.

The different trend observed in organic solvents and ionic liquids indicated that they deactivate the enzymes following different mechanisms. Compared to the functional changes, however, the structural changes in the presence of increasing concentrations of solvents were minimal, which is in agreement with the literature (**Russell** et al., 1992).

The water-free media were not appropriate for BAL and BFD*H281A* because the cofactor is an additional component which makes the system even more complex. Most examples of successful application in water-free media are with enzymes which do not require cofactors (**Walker** and **Bruce**, 2004).

Clearly, BAL and BFD*H281A* did not work optimally in aqueous buffer. However, the experiments with the enzymes in water/solvent mixtures showed that there is a space for improvement. In presence of DMSO, for example, BAL - a relatively unstable enzyme - was significantly stabilized, whereas BFD*H281A* - which has a very low activity, was activated. The choice of appropriate solvent can greatly improve the enzymes' performance and expand their potential.

VI. REFERENCES

Andersson, R.E. 1980. Microbial lipolysis at low temperatures, *Appl Environ Microbiol*, 39 (1), 36-40.

Bell, G., Halling, P.J., Moore, B.D., Partridge, J., Rees, D.G. 1995. Biocatalyst behaviour in low-water content, *Trends Biotechnol*, 13, 468-473.

Bell, G., Janssen, A.E.M., Halling, P.J. 1997. Water activity fails to predict critical hydration level for enzyme activity in polar organic solvents: Interconversion of water concentrations and activities, *Enzyme Microb Technol*, 20 (6), 471-477.

Bell, G., Halling, P.J., May, L., Moore, B.D., Robb, D.A., Ulijn, R., Valivety, R.H. 2001. Enzymes in non-aqueous solvents: Methods and protocols. 106-126. Vulfson, E.N., Halling, P.J., Holland, H.L. Methods for measurement control of water in nonaqueous biocatalysis, Totowa, Humana Press Inc. (textbook).

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem*, 72, 248-254.

Broering, J.M., Bommarius, A.S. 2005. Evaluation of Hofmeister effects on the kinetic stability of proteins, *J Phys Chem B*, 109, 20612-20619.

Bruins, M.E., Janssen, A.E., Boom, R.M. 2001. Thermozymes and their applications: a review of recent literature and patents, *Appl Biochem Biotechnol*, 90 (2), 155-186.

Carrea, G., Ottolina G., Riva S. 1995. Role of solvents in the control of enzyme selectivity in organic media, *Trends Biotechnol*, 13 (2), 63- 70.

Castro, G.R., Knubovets, T. 2003. Homogeneous biocatalysis in organic solvents and water-organic mixtures, *Crit Rev Biotechnol*, 23 (3), 195-231.

Chakraborty, S., Nemeria, N., Yep, A., McLeish, M., Kenyon, G.L., Jordan, F. 2008. Mechanism of benzaldehyde lyase studied via thiamine diphosphate-bound intermediates and kinetic isotope effects, *Biochemistry*, 47 (12), 3800-3809.

Celebi, N., Yildiz, N., Demir, A.S., Calimli, A. 2007. Enzymatic synthesis of benzoin in supercritical carbon dioxide, *J Supercrit Fluid*, 41, 386-390.

Cowan, D.A. 1997. Relationships between enzyme stability, activity and conformational mobility in organic solvents, *Comp Biochem Physiol*, 118 A (3), 429-438.

Dai, L., Klibanov A. M. 1999. Striking activation of oxidative enzymes suspended in nonaqueous media, *Proc Natl Acad Sci, USA*, 96 (17), 9475-9478.

Davies, D.L., Kandola, S.K., Patel, R.K. 2004. Asymmetric cyclopropanation in ionic liquids: effect of anion and impurities, *Tetrahedron: Asymmetry*, 15, 77-80.

De Diego, T., Lozano, P., Gmouh, S., Vaultier, M., Iborra, J.L. 2004. Fluorescence and CD spectroscopic analysis of the alpha-chymotrypsin stabilization by the ionic liquid, 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide, *Biotechnol Bioeng*, 88 (7), 916-924.

DeLano, W.L. 2002. The PyMOL Molecular Graphics System, http://www.pymol.org, San Carlo, CA

Demir, A.S., Dünnwald, T., Iding H., Pohl, M., Müller, M. 1999. Asymmetric benzoin reaction catalyzed by benzoylformate decarboxylase, *Tetrahedron: Asymmetry*, 10 (24), 4769-4774.

Demir, S., Pohl, M., Janzen, E., Müller, M. 2001. Enantioselective synthesis of hydroxy ketones through cleavage and formation of acyloin linkage. Enzymatic kinetic resolution via C–C bond cleavage, *J Chem Soc Perkin Trans*, 1, 633-635.

Demir, A.S., Şeşenoglu, Ö., Eren, E., Hosrik, B., Pohl, M., Janzen, E., Kolter, D., Feldmann, R., Dünkelmann, P., Müller, M. 2002. Enantioselective synthesis of alpha-hydroxy ketones via benzaldehyde lyase-catalyzed C-C bond formation reaction, *Adv Synth Catal*, 344 (1), 96-103.

Demir, A.S., Şeşenoglu, Ö., Dünkelmann, P., Müller, M. 2003. Benzaldehyde Lyase-Catalyzed Enantioselective Carboligation of Aromatic Aldehydes with Mono- and Dimethoxy Acetaldehyde, *Org Lett*, 5 (12), 2047-2050.

Demirjian, D.C., Moris-Varas, F., Cassidy, C.S. 2001. Enzymes from extremophiles, *Curr Opin Chem Biol*, 5 (2), 144-151.

Dhawan, S., Kaur, J. 2007. Microbial mannanases: an overview of production and applications, *Crit Rev Biotechnol*, 27 (4), 197-216.

Domínguez de María, P., Stillger, T., Pohl, M., Wallert, S., Drauz, K., Gröger, H., Trauthwein, H., Liese, A. 2006. Preparative enantioselective synthesis of benzoins and *(R)*-2-hydroxy-1-phenylpropanone using benzaldehyde lyase, *J Mol Catal B: Enzym*, 38, 43-47.

Domínguez de María, P., Pohl, M., Gocke, D., Gröger, H., Trauthwein, H., Stillger, T., Müller, M. 2007. Asymmetric synthesis of aliphatic 2-hydroxy ketones by enzymatic carboligation of aldehydes, *Eur J Org Chem*, 18, 2940-2944.

Domínguez de María, P., Stillger, T., Pohl, M., Kiesel, M., Liese, A., Gröger, H., Trauthwein, H. 2008. Enantionselective C-C Bond Ligation Using Recombinant Escherichia coli-Whole-Cell Biocatalysts, *Adv Synth Catal*, 350 (1), 165-173.

Ducret, A., Trani, M., Lortie, R. 1998. Lypase-catalysed enantioselective esterification of ibuprofen in organic solvents under controlled water activity, *Enzyme Microb Technol*, 22, 212-216.

Dünkelmann, P., Kolter-Jung, D., Nitsche, A., Demir, A.S., Siegert, P., Lingen, B., Baumann, M., Pohl, M., Müller, M. 2002. Development of a donor-acceptor concept for enzymatic cross-coupling reactions of aldehydes: the first asymmetric cross-benzoin condensation, *J Am Chem Soc*, 124 (41), 12084-12085.

Dünnwald, T., Demir, A.S., Siegert, P., Pohl, M., Müller, M. 2000. Enantioselective synthesis of (*S*)-2-hydroxypropanone derivatives by benzoylformate decarboxylase catalyzed C-C bond formation, *Eur J Org Chem*, 2161-2170.

Eckstein, M.F. 2004. Alcohol dehydrogenase catalysed reductions in non-conventional media, Universität Rostock (dissertation).

Erixon, K.M., Dabalos, C.L., Leeper, F.J. 2007. Inhibition of pyruvate decarboxylase from *Z. mobilis* by novel analogues of thiamine diphosphate: investigating pyrophosphate mimics, *Chem Commun*, 960-962.

Faber, K. 1997. Biotransformations in Organic Chemistry, Springer, Berlin, Heidelberg, New York (textbook).

Fiedler, E., Thorell, S., Sandalova, T., Golbik, R., König, S., Schneider, G. 2002. Snapshot of a key intermediate in enzymatic thiamin catalysis: Cristal structure of the α-carbaion of

(*α,β*- dihydroxyethyl)-thiamin diphosphate in the active site of transkelotase from *Saccaromyces cerevisiae*, PNAS, 99 (2), 591-595

Filho, M.V., Stillger, T., Müller, M., Liese, A., Wandrey, C. 2003. Is log P a convenient criterion to guide the choice of solvents for biphasic enzymatic reactions?, *Angew Chem Int Ed*, 42 (26), 2993-2996.

Fontes, N., Almeida M., Peres C., Garcia S., Grave J., Aires-Barros M.R., Soates C., Carbal J., Maycock C., Barreiros S. 1998. Cutinase activity and enantioselectivity in supercritical fluids, *Ind Eng Chem Res,* 37, 3189-3194.

Frank, R.A.W., Leeper, F.J., Luisi, B.F. 2007. Structure, mechanism and catalytic dualisti of thiamine-dependent enzymes, Cell Mol Life Sci, 64, 892-905.

Garza-Ramos, G., Fernandez-Velasco D.A., Ramirez, L., Shoshani, L., Darszon, A., Tuena de Gomez-Puyou, M., Gomez-Puyou, A. 1992. Enzyme activation by denaturants in organic solvent systems with low water content, *Eur J Biochem*, 205, 509-517.

Gocke, D., Walter, L., Gauchenova, E., Kolter, G., Knoll, M., Berthold, C.L., Schneider, G., Pleiss, J., Müller, M., Pohl, M. 2008[a]. Rational protein design of ThDP-Dependent enzymes - engineering stereoselectivity, *Chembiochem*, 9, 406-412.

Gonzalez, B., Vicuna, R. 1989. Development of a donor-acceptor concept for enzymatic cross-coupling reactions of aldehydes: the first asymmetric cross-benzoin condensation, *J Bacteriol*, 171 (5), 2401-2405.

Gorman, L.A.., Dordick, J.S. 1992. Organic solvents strip water off enzymes, *Biotechnol Bioeng*, 39 (4), 392-397.

Grant, W.D. 2004. Life at low water activity, *Philos T Royal Soc B Biol Sci*, 359 (1448), 1249-1267.

Gupta, M.N. 1992. Enzyme function in organic solvents, *Eur J Biochem*, 203, 25-32.

Gupta, M.N., Roy, I. 2004. Enzymes in organic media: Forms, functions and applications, *Eur J Biochem*, 271 (13), 2575-2583.

Hammond, D.A., Karel, M., Klibanov, A.M., Krukonis, V.J. 1985. Enzymatic reactions in supercritical gases, *Appl Biochem Biotech*, 11 (5), 393-400.

Hartmann, T., Meyer, H., Scheper, T. 2001. The enantioselective hydrolysis of 3-hydroxy-5-phenyl-4-pentenoicacidethylester in supercritical carbon dioxide using lipases, *Enzyme Microb Tech*, 28 (7), 653-660.

Hasson, M.S.., Muscate, A., McLeish M.J., Polovnikova, L.S., Gerlt, J.A., Kenyon, G.L., Petsko, G.A., Ringe, D. 1998. The crystal structure of benzoylformate decarboxylase at 1.6 A resolution: diversity of catalytic residues in thiamine diphosphate-dependent enzymes, *Biochemistry*, 37 (28), 9918-9930.

Hegeman, G.D. 1970. Benzoylformate decarboxylase (*Pseudomonas putida*), *Methods Enzymol*, 17A, 674-678.

Hildebrand, F., Kühl, S., Pohl, M., Vasic-Racki, D., Müller, M., Wandrey, C., Lütz, S. 2007. The production of *(R)*-2-hydroxy-1-phenyl-propan-1-one derivatives by benzaldehyde lyase from *Pseudomonas fluorescens* in a continuously operated membrane reactor, *Biotechnol Bioeng*, 96 (5), 835-843.

Hinrichsen, I., Gomez. R., Vicuna, R. 1994. Cloning and sequencing of the gene encoding benzaldehyde lyase from *Pseudomonas fluorescens* biovar I, *Gene*, 144, 137-138.

Hobbs, H.R., Thomas, N.R. 2007. Biocatalysis in Supercritical Fluids, in Fluorous Solvents, and under Solvent-Free Conditions, *Chem Rev*, 107 (6), 2786-2820.

Hobbs, H.R., Kirke, H.M., Poliakoff, M., Thomas, N.R. 2007. Homogeneous biocatalysis in both fluorous biphasic and supercritical carbon dioxide systems, *Angew Chem Int Ed*, 46, 1-5.

Hofmeister, F., 1888. Zur Lehre von der Wirkung der Salze II, *Arch Exp Pathol Pharmacol*, 24, 247-260.

Iding, H., Dünnwald T., Greiner, L., Liese, A., Müller, M., Siegert, P., Grötzinger, J., Demir, A.S., Pohl, M. 2000. Benzoylformate decarboxylase from *Pseudomonas putida* as stable catalyst for the synthesis of chiral 2-hydroxy ketones, *Chem Eur J*, 6 (8), 1483-1495.

Ikushima, Y., Saito, N., Arai, M., Blanch, H.W. 1995. Activation of a lipase triggered by interactions with supercritical carbon dioxide in the near-critical region, *Phys Chem*, 99 (2), 8941-8944.

Janzen, E. 2002. Die Benzaldehydlyase aus *Pseudomonas fluorescenzens*: Biochemische Charakterisierung und die Untersuchung von Struktur-Funktionsbeziehungen, Heinrich-Heine Universität Düsseldorf (dissertation).

Janzen, E., Müller, M., Kolter-Jung, D., Kneen, M.M., McLeish, M.J, Pohl, M. 2006. Characterization of benzaledyde lyase from *Pseudomonas fluorescens* - a versatile enzyme for asymmetric C-C bond formation, *Bioorg Chem*, 34, 345-361.

Jordan, F., Sergienko, E., Nemeria, N., Liu, M., Wang, J., Guo, F., Furey, W.F. 2000. Structure-function studies in thiamin diphosphate-dependent 2-oxo acid decarboxylating enzymes, *Vestnik Moskovskovo Universiteta*, *Khimiya*, 41 (6), suppl., 62-66.

Kaar, J.L ., Jesionowski, A.M., Berberich, J.A., Moulton, R., Russel, A. 2003. Impact of ionic liquid physical properties on lipase activity and stability, *J Am Chem Soc,* 125, 4125-4131.

Kamat, S.V., Iwaskewycz, B., Beckman, E.J., Russell, A.J. 1993. Biocatalytic synthesis of acrylates in supercritical fluids: tuning enzyme activity by changing pressure, *Proc Natl Acad Sci USA*, 90, 2940-2944.

Karmee, S.K., Casiraghi, L., Greiner, L. 2007. Technical aspects of biocatalysis in non-CO2-based supercritical fluids, *Biotechnol J*, 3 (1), 104-111.

Ke, T., Klibanov, A.M. 1998. On enzymatic activity in organic solvents as a function of enzyme history, *Biotechnol Bioeng*, 57 (6), 746-750.

Kermasha, S., Bao, H., Bisarkowski, B. 2001. Biocatalysis of tyrosinase using catechin as substrate in selected organic solvent media, *J Mol Catal B: Enzym*, 11, 929-938

Khmelnitsky, Y.L., Mozhaev, V.V., Belova, A.B., Sergeeva, M.V., Martinek, K. 1991. Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis, *Eur J Biochem*, 198, 31-41.

Kirk, O., Borchert, T.V., Fuglsang, C.C. 2002. Industrial enzyme applications, *Curr Opin Biotech*, 13 (4), 345-351.

Klibanov, A.M. 2000. Answering the question: Why did biocatalysis in organic media not take off in the 1930s?, *Trends Biotechnol*, 18 (3), 85-86.

Klibanov, A.M. 2001. Improving enzymes by using them in organic solvents, *Nature*, 409, 241-246.

Knoll, M., Müller, M., Pleiss, J., Pohl, M. 2006. Factors mediating activity, selectivity, and substrate specificity for the thiamine diphosphate-dependent enzymes benzaldehyde lyase and benzoylformate decarboxylase, *ChemBioChem*, 7, 1928-1934.

Korz, D.J., Rinas, U., Hellmuth, K., Sanders, E.A., Deckwer, W.-D. 1995. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*, *J Biotechnol*, 39, 59-65.

Krishna, S.H. 2002. Developments and trends in enzyme catalysis in nonconventional media, *Biotechnol Adv*, 20, 239-267.

Kühl, S., Zehentgruber, D., Pohl, M., Müller, M., Lütz, S. 2007. Process development for enzyme catalysed asymmetric C-C-bond formation, *Chem Eng Sci*, 62, 5201-5205.

Lee, S.H., Ha, S.H., Lee, S.B., Koo, Y.-M. 2006. Adverse effect of chloride impurities on lipase-catalysed transesterifications in ionic liquids, *Biotechnol Lett*, 28, 1335-1339.

Lemos, M.A., Oliveira, J.C., Saraiva, J.A. 2001. Effects of water content on the thermal inactivation kinetics of horseradish peroxidase freeze dried from alkaline pH, *Food Sci Technol Int*, 7 (5), 393-398.

Lingen, B., Grötzinger, J., Kolter, D., Kula, M.-R., Pohl, M. 2002. Improving the carboligase activity of benzoylformate decarboxylase from *Pseudomonas putida* by a combination of directed evolution and site-directed mutagenesis, *Protein Eng*, 15 (7), 585-593.

Lingen, B., Kolter-Jung, D., Dünkelmann, P., Feldmann, R., Grötzinger, J., Pohl, M., Müller, M. 2003. Alteration of the Substrate Specificity of Benzoylformate Decarboxylase from *Pseudomonas putida* by Directed Evolution, *Chembiochem*, 4, 721-726.

Lozano, P., De Diego, T., Carrie, D., Vaultier, M., Iborra, J.L. 2003. Lipase catalysis in ionic liquids ans supercritical carbon dioxide at 150 degrees C., *Biotechnol Prog*, 19 (2), 380-382.

Lozano, P., De Diego, T., Gmouh, S., Vaultier, M., Iborra, J.L. 2004. Criteria to desing green enzymatic processes in ionic liquids/supercritical carbon dioxide systems, *Biotechnol Prog*, 20 (3), 661-669.

Mase, N., Sako, T., Horikawa,, Y., Takabe, K. 2003. Novel strategic lipase-catalyzed asymmetrization of 1,3-propanediacetate in supercritical carbon dioxide, *Tetrahedron Lett*, 44, 5175-5178.

Matsuda, T., Watanabe, K., Harada, T., Nakamura, K. 2004. Enzymatic reactions in supercritical CO_2: carboxylation, asymmetric reduction and esterification, *Catal Today*, 96, 103-111.

Mikolajek, R., Spiess, A.C., Pohl, M., Lamare, S., Büchs, J. 2007. An activity, stability and selectivity comparison of propioin synthesis by thiamine diphosphate-dependent enzymes in a solid/gas bioreactor, *Chembiochem*, 8, 1063-1070.

Miroliaei, M., Nemat-Gorgani, M. 2002. Effect of organic solvents on stability and activity of two related alcohol dehydrogenases: a comparative study, *Int J Biochem Cell Biol*, 34, 169-175.

Mosbacher, T.G., Müller, M., Schulz, G.E. 2005. *FEBS J*, 272 (23), "Structure and mechanism of the ThDP-dependent benzaldehyde lyase from *Pseudomonas fluorescens*", 6067-6076.

Nemeria, N., Korotchkina, L., McLeish, M., Kenyon. G.L., Patel, M.S., Jordan, F. 2007. Elucidation of the chemistry of enzyme-bound thiamin diphosphate prior to substrate binding: defining internal equilibria among tautomeric and ionisation states, *Biochemistry*, 46 (37), 10739-10744.

Nielsen, J.E., McCammon, J.A. 2003. Calculating pKa values in enzyme active sites, *Protein Sci*, 12, 1894-1901.

Pilissao, C., Nascimento M.G. 2006. Effects of organic solvents and ionic liquids on the aminolysis of *(R,S)*-methyl mandelate catalyzed by lipases, *Tetrahedron: Asymmetry, 17,* 428-433.

Pohl, M., Lingen, B., Müller, M. 2002. Thiamine-diphosphate dependent enzymes: new aspects of asymmetric C-C bond formation, *Chem Eur J*, 8 (23), 5288- 5295.

Polovnikova, E.S., McLeish, M.J., Sergienko, E.A., Burgner, J.T., Anderson, N.L., Bera, A.K., Jordan, F., Kenyon, G.L., Hasson, M.S. 2003. Structural and kinetic analysis of catalysis by a thiamine diphosphate-dependent enzyme, benzoylformate decarboxylase, *Biochemistry*, 25 (42), 1820-1830.

Rantakylä, M., Aaltonen, O. 1994. Enatioselective esterification of ibuprofen in supercritical carbon dioxide by immobilizer lipase, *Biotechnol Lett*, 16 (8), 825-830.

Rariy, R.V., Klibanov A.M. 2000. On the relationship between enzymatic enantioselectivity in organic solvents and enzyme flexibility, *Biocatal Biotransform*, 18, 401-407.

Rezaei, K., Temelli, F., Jenab, E. 2007. The effects of pressure and temperature on the enzymatic reactions in supercritical fluids, *Biotechnol Adv*, 25 (3), 272-280.

Rodriguez Couto, S., Toca Herrera, J.L. 2006. Industrial and biotechnological applications of laccases: A review, *Biotechnol Adv*, 24 (5), 500-513.

Rupley, J.A., Careri, G. 1991. Protein hydration and function, *Adv Protein Chem*, 41, 37-172.

Russell, A.J., Chatterjee, S., Bambot, S. 1992. Mechanistic enzymology in non-aqueous media, *Pure Appl Chem*, 64 (8), 1157-1163.

Schmid, F.X. 1989. Spectral probes of conformation: in: protein structure - a practical approach, (T. E. Creighton, ed.), IRL press Oxford, New York Tokio (textbook), p. 269.

Scopes, R.K. 1993. Protein Purification: Principles and Practice, Springer-Verlag New York, LLC (textbook).

Seddon, K.R., Stark, A., Torres, M.J. 2000. Influence of chloride, water, and organic solvents on the physical properties of ionic liquids, *Pure Appl Chem*, 72 (12), 2275-2287.

Sehgal, A.C., Tompson, R., Cavanagh, J., Kelly, R.M. 2002. Structural and catalytic response to temperature and cosolvents of carboxylesterase EST1 from the extremely thermoacidophilic archeon *Sulfolobus solfataricus* P1, *Biotechnol Bioeng*, 80 (7), 785-793.

Serdakowski, A.L., Dordick, J.S. 2007. Enzyme activation for organic solvents made easy, *Trends Biotechnol*, 26 (1), 48-54.

Siegert, P. 2000. Vergleichende Characterisierung der Decarboxylase- und Carboligasereaktion der Benzoylformiatdecarboxylase aus Pseudomonas putida und der Pyrovatdecarboxylase aus Zymomonas mobilis mittels gerichteter Mutagenese, Heinrich-Heine Universität Düsseldorf (dissertation).

Siegert, P., McLeish, M.J., Baumann, M., Iding, H., Kneen, M.M., Kenyon, G.L., Pohl, M. 2005. Exchanging the substrate specificities of pyruvate decarboxylase from Zymomonas mobilis and benzoylformate decarboxylase from Pseudomonas putida, *Protein Eng Des Sel*, 18 (7), 345-357.

Stillger, T. 2004. Enantioselektive C-C-Knüpfung mit Enzymen, Charakterisierung und reaktionsthechnische Bearbeitung der Benzaldehydlyase aus *Pseudomonas fluorescens* Biovar I, Rheinische Friedrich Wilhelms-Universität Bonn (dissertation).

Stillger, T., Pohl, M., Wandrey, C., Liese, A. 2006. Reaction engineering of benzaldehyde lyase catalyzing enantioselective C-C bond formation, *Org Proc Res Dev*, 10 (6), 1172-1177.

Summers, C.A., Flowers, R.A. 2000. Protein renaturation by the liquid organic salt ethylammonium nitrate, *Protein Sci*, 9, 2001-2008.

Sym, E.A. 1933. Über die Esterasewirkung III, *Biochem Z*, 258-304.

Tawaki, S., Klibanov, A.M. 1992. Inversion of enzyme enantioselectivity mediated by the solvent, *J Amer Chem Soc*, 114, 1882-1884.

Tittmann, K., Golbik, R., Uhlemann, K., Khailova, L., Schneider, G., Patel, M., Jordan, F., Chipman, D.M., Duggleby, R.G., Hübner, G. 2003. NMR analysis of covalent intermediates in thiamin diphosphate enzymes, *Biochemistry*, 42, 7885-7891.

Tsou, A.Y., Ransom, S.C., Gerlt, J.A., Buechter, D.D., Babbit, P.C., Kenyon, G.L. Mandelate pathway of *Pseudomonas putida*: sequence relationships involving mandelate racemase, *(S)*-mandelate dehydrogenase, and benzoylformate decarboxylase and expression of benzoylformate decarboxylase in *Escherichia coli*, 1990. *Biochemistry*, 29, 9856-9862.

Valivety, R.H., Halling, P.J., Macrae, A.R. 1992[a]. Reaction rate with suspended lipase catalyst shows similar dependence on water activity in different organic solvents, *Biochim Biophis Acta*, 1118, 218-222.

Valivety, R.H., Halling, P.J., Peilow, A.D., Macrae, A.R. 1992[b]. Lipases from different sources vary widely in dependence of catalytic activity on water activity, *Biochim Biophys Acta*, 1122, 143-146.

van Rantwijk, F., Sheldon, R.A. 2007. Biocatalysis in ionic liquids, *Chem Rev*, 107, 2757-2785

Vivian, J.T., Callis, P.R. 2001. Mechanism of tryptophan fluorescence shifts in proteins, *Biophys J*, 80 (5), 2093-2109.

Volkin, D.B., Staubli, A., Langer, R., Klibanov, A.M. 1991. Enzyme thermoinactivation in anhydrous organic solvents, *Biotechnol Bioeng*, 37, 843-853.

Vuppugalla R., Chang, S.Y., Zhang H., Marathe, P.H., Rodrigues, D.A. 2007. Effect of commonly used organic solvents on the kinetics of cytochrome P450 2B6- and 2C8-dependent activity in human liver microsomes, *Drug Metab Dispos*, 35 (11), 1990-1995.

Walker, A. J., Bruce, N.C. 2004. Cofactor-dependent enzyme catalysis in functionalized ionic liquids, *Chem Commun*, 2570-2571.

Wilcocks, R., Ward, O.P., Collins, S., Dewdney, N.J., Hong, Y., Prosen, E. 1992. Acyloin formation by benzoylformate decarboxylase from *Pseudomonas putida*, *Appl Environ Microbiol*, 58 (5), 1699-1704.

Williams, J.R., Clifford, A.A., Al-Saidi, S.H.R. 2002. Supercritical fluids and their applications in biotechnology and related areas, *Mol Biotechnol*, 22 (3), 263-286.

Wu, J.C., Lee, S.S., Mahmood, M.M.B., Chow, Y., Talukder, M.M.R., Choi, W.J. 2007. Enhanced activity and stability of immobilized lipases by treatment with polar solvents prior to lyophilization, *J Mol Catal B: Enzym*, 45, 108-112.

Xu, K., Klibanov, A.M. 1996. pH control of the catalytic activity of cross-linked enzyme crystals in organic solvents, *J Am Chem Soc*, 118 (41), 9815-9819.

Yang, L., Jonatan, S., Dordick, S., Garde, S. 2004. Hydration of enzymes in nonaqueous media is consistent with solvent dependence of its activity, *Biophys J*, 87, 812-821.

Yang, Z., Pan, W. 2005. Ionic liquids: green solvents for nonaqueous biocatalysis, *Enzyme Microb Technol*, 37 (1), 19-28.

Yang, Z., Yue, Y.-J., Xing, M. 2007. Tyrosinase activity in ionic liquids, *Biotechnol Lett*, 30, 153-158.

Zaks, A., Klibanov, A.M. 1985. Enzyme-catalyzed processes in organic solvents, *Proc Natl Acad Sci*, 82, 3192-3196.

Zaks, A., Klibanov, A.M. 1986. Substrate specificity of enzymes in organic solvents vs. water is reversed, *J Am Chem Soc*, 108 (10), 2767-2768.

Zavrel, M., Schmidt, T., Michalik, C., Ansorge-Schumacher, M., Marquardt, W., Büchs, J., Spiess, A.C. 2008. Mechanistic kinetic model for symmetric carboligations using benzaldehyde lyase, *Biotechnol Bioeng*, 101 (1), 27-38.

Zhao, H., Campbell, S., Jackson, L., Song, Z., Olubajo, O. 2006[a]. Hofmeister series of ionic liquids: kosmotropic effect of ionic liquids on the enzymatic hydrolysis of enantiomeric phenylalanine methyl ester, *Tetrahedron: Asymmetry*, 17, 377-383.

Zhao, H., Campbell, S., Solomon, J., Song, Z., Olubajo, O. 2006[b]. Improving the enzyme catalytic efficiency using ionic liquids with kosmotropic anions, *Chinese J Chem*, 24, 580-584.

Zhao, H., Olubajo, O., Song, Z., Sims, A.L., Person, T.E., Lawal, R.A., Holley, L.A. 2006[c]. Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions, *Bioorg Chem*, 34, 15-25.

VII. APPENDIX

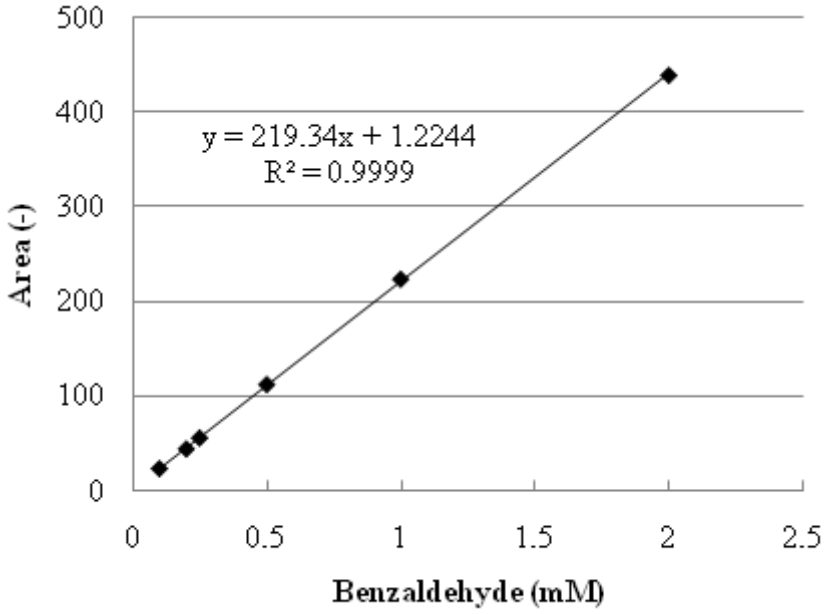


Figure A1: Calibration curve for determination of benzaldehyde concentration. Data were obtained using the HPLC procedure described in **II.4.4.4.**

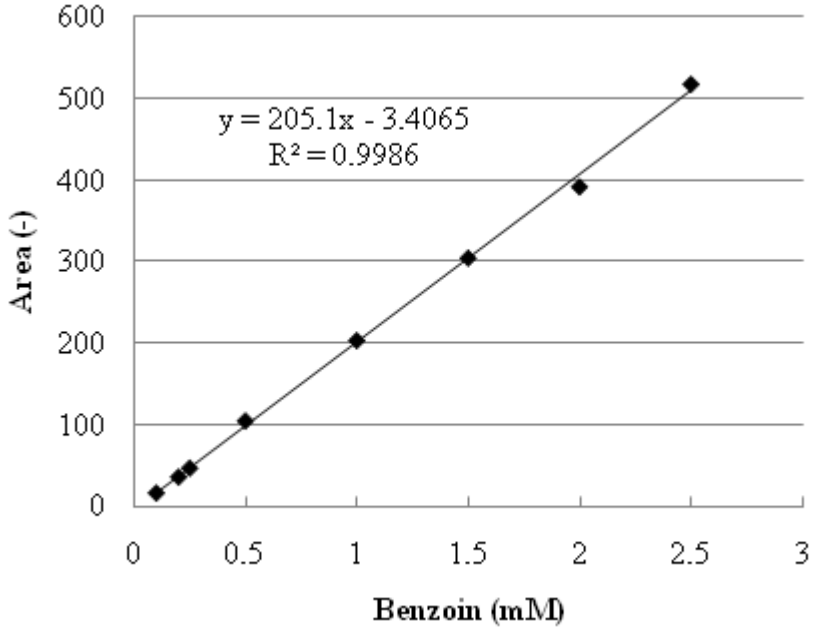


Figure A2: Calibration curve for determination of benzoin concentration. Data were obtained using the HPLC procedure described in **II.4.4.4.**

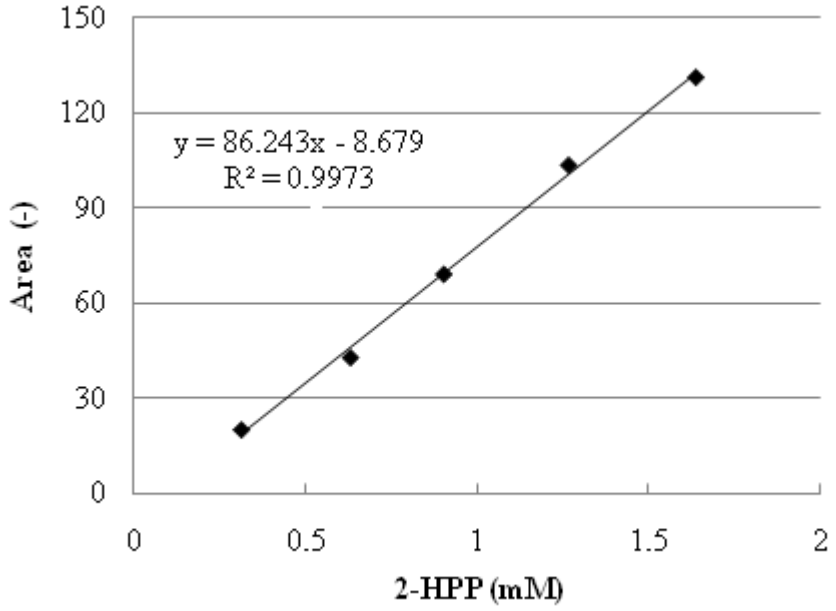


Figure A3: Calibration for determination of 2-HPP concentration. Data were obtained using the HPLC procedure described in **II.4.7.**

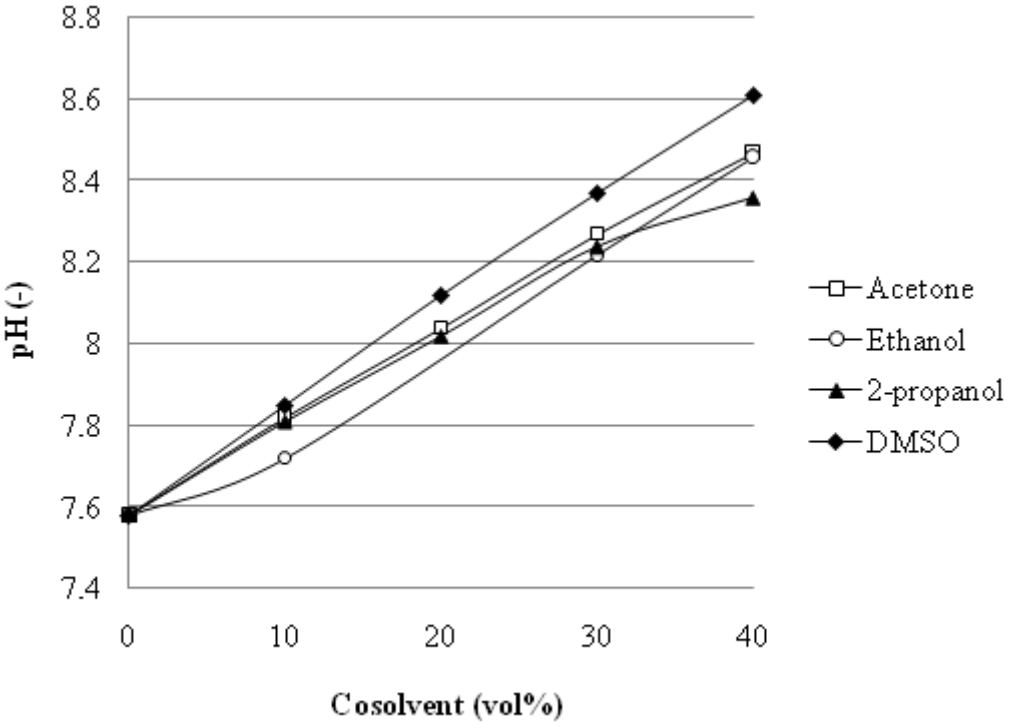


Figure A4: Shift of apparent pH in organic solvents. pH was measured in mixtures of cosolvents and 50 mM potassium phosphate buffer (starting pH 7.6), at room temperature.

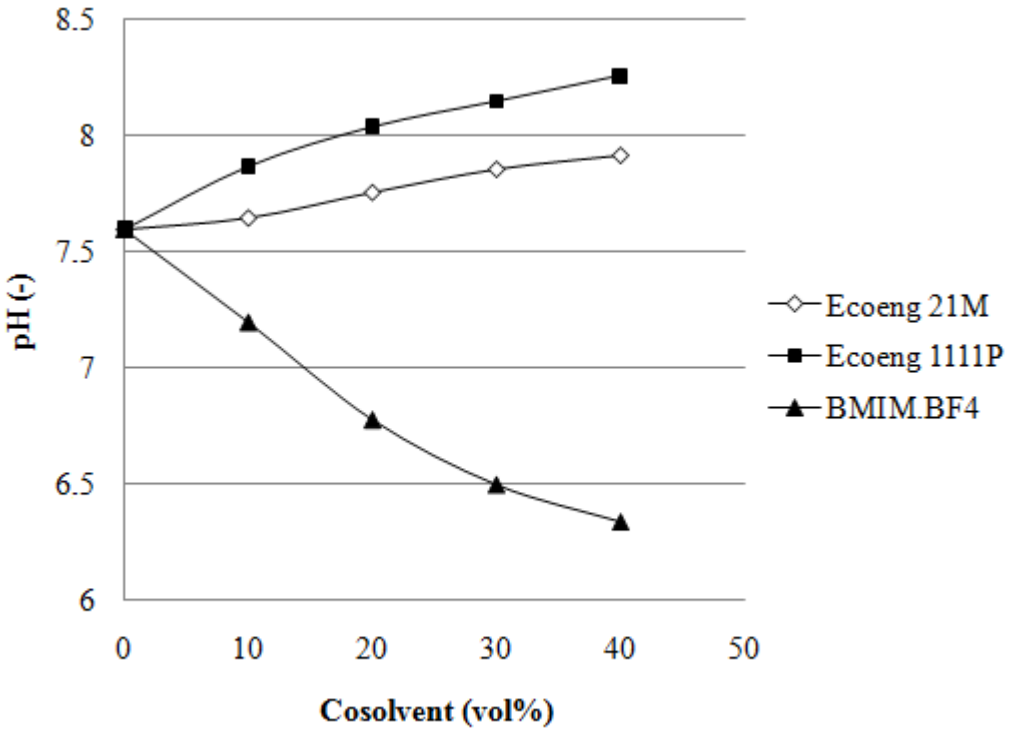


Figure A5: Shift of apparent pH in ionic liquids. pH was measured in mixtures of ILs and 50 mM potassium phosphate buffer (starting pH 7.6), at room temperature.

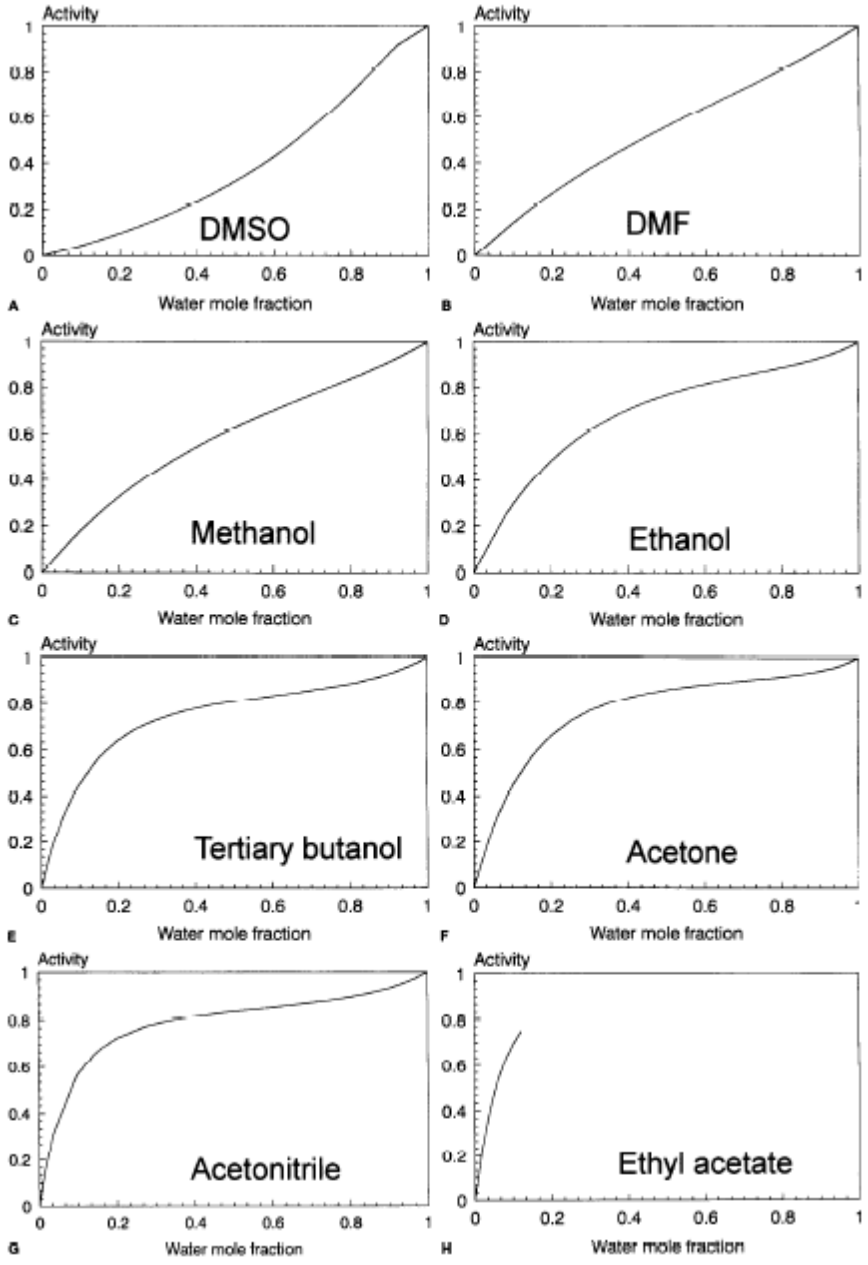


Figure A6: Calculated water activity in water/organic solvent mixtures (Bell et a., 1997).

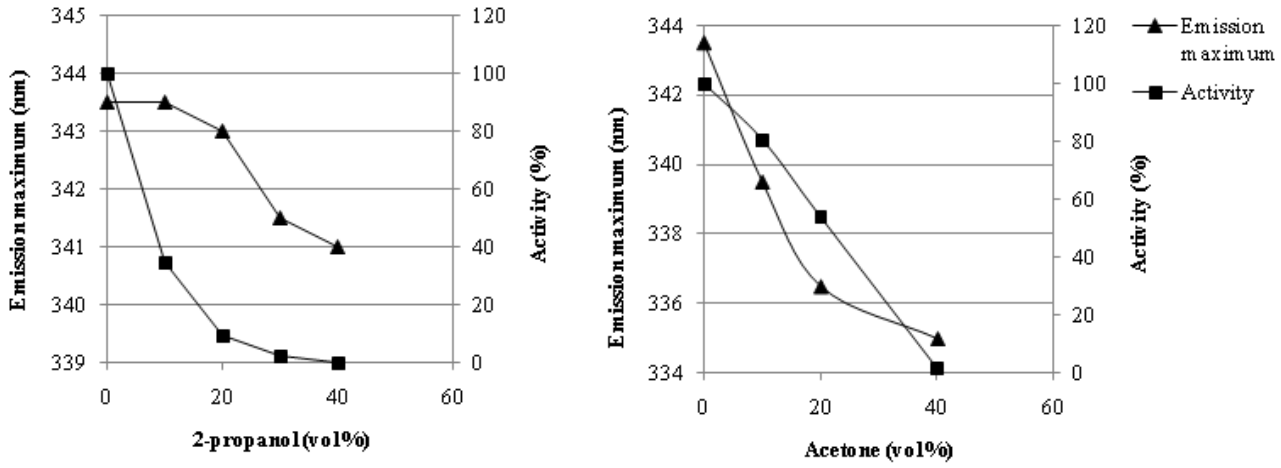


Figure A7: Tryptophan fluorescence and activity of BAL in 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) containing increasing concentrations of 2-propanol and acetone. pH adjusted to 8.

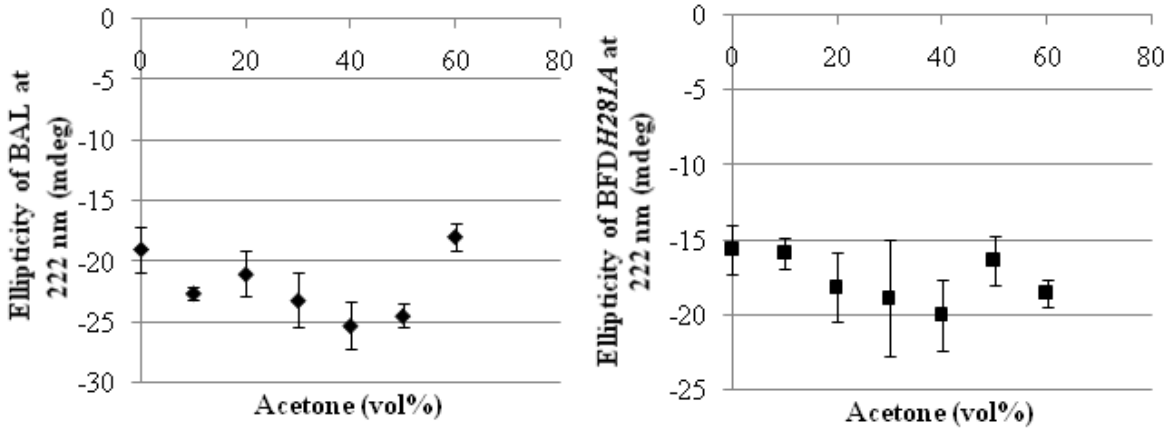


Figure A8: Ellipticity of BAL and BFD*H281A* in 50 mM potassium phosphate buffer (2.5 mM $MgSO_4$, 0.5 mM ThDP) containing increasing concentrations of acetone after 5 min incubation at 30°C. pH adjusted to 8 (BAL) and 6.5 (BFD*H281A*). Protein concentration 0.5 mg/ml. Error bars indicate SD of two independent experiments.

Table A1: Stock solutions for high cell density fermentation.
Quantity of ingredients and type of sterilization are indicated.

Stock solutions:

Autoclave:

HCD Batch	(g)	(g/l)
NH_4Cl	3	2
$(NH_4)_2SO_4$	30	20
KH_2PO_4	195	130
K_2HPO_4	150	100
NaH_2PO_4-H_2O	90	60
Yeast extract	45	30
Final volume[3] (ml)	1500	

Autosterile:

Trace elements	(g)	(g/l)
$CaCl_2$-$2H_2O$	10	10
$ZnSO_4$-$7H_2O$	0.5	0.5
$CuCl_2$-$2H_2O$	0.25	0.25
$MnSO_4$-H_2O	2.5	2.5
$CoCl_2$-$6H_2O$	1.75	1.75
H_3BO_3	0.125	0.125
$AlCl_3$-$6H_2O$	2.5	2.5
Na_2MoO_4-$2H_2O$	0.5	0.5
$FeSO_4$-$7H_2O$	10	10
Final volume[1] (ml)	1000	

Autoclave:

HCD Feed[4]	(g)	(g/l)
HCD Batch	500 ml	
Yeast extract	75	180

Autoclave:

$MgSO_4$ solution	(g)	(g/l)
$MgSO_4$-$7H_2O$	60	200
Final volume[1] (ml)	300 (250+50 alliquots)	

Sterile filtrate:

Ampicillin	(g)	(g/l)
Ampicillin	2	200
Kanamycin	0.75	75
Final volume[1] (ml)	10	

Autoclave:

Glucose solution Batch	(g)	(g/l)
Glucose-H_2O	20	400
Final volume[1] (ml)	10	

Sterile filtrate:

Vitamin solution[5]	(g)	(g/l)
Riboflavin (B_2)	0.05	0.1
Thiamine-HCl (B_1)	5	10
Nicotine acid	0.25	0.5
Pyridoxine-HCl (B_6)	0.25	0.5
Ca-Panthotenate	0.25	0.5
Biotin	0.0005	0.001
Folic acid	0.001	0.002
Cyanocobalamin (B_{12})	0.005	0.01
Final volume[1] (ml)	1000	

Sterile filtrate:

IPTG[6]	(g)	(g/l)
IPTG	7	1
Final volume[1] (ml)	30	

Sterile fitrate:

Thiamine solution	(g)	(g/l)
Thiamine HCl (B_1)	6	200
Final volume[1] (ml)	50	

[3] With distilled water
[4] Divide the HCD Batch into 1000+500 ml, add 75 g glucose to the 500 ml portion to prepare HCD Feed
[5] Divide vitamin stock solutions in 50+25 ml portions and keep in a freezer
[6] Keep in a freezer until induction

Table A2: Composition of batch and feed media for high cell density fermentation

Media:

Batch medium	V (ml)
HCD Batch	1000
Antifoam	3
Glucose solution	50
$MgSO_4$ solution	50
Vitamin solution	50
Trace elements	40
Thiamine Solution	5
Inoculum	100
Distilled water	8700
Total	10000

Feed medium	V (ml)
HCD Feed	500
Glucose	4180
$MgSO_4$	250
Vitamin	25
Trace elements	20
Thiamine	25
Antifoam	2
Total	5002

Table A3: Solubility of benzoin in water-immiscible solvents at 30°C.

Saturated solutions of analytes were mixed with different organic solvents for 24 h at 30ºC. After centrifugation at 13,000 rpm for 1h, the excess amount of analytes was discarded, and the rest centrifuged a second time. Amounts of benzaldehyde and benzoin were determined using the HPLC procedure described in **II.4.4.4**. Solubility in water and 30 vol% DMSO given for comparison.

Solvent	Solubility (mM)
Water	0.70
Hexane	1.09
Petroleum ether	1.45
30 vol% DMSO	2.78
MTBE	24.41
Toluene	44.20

Table A4: Solubility of benzaldehyde and benzoin in different concentrations of water-miscible organic solvents at 30°C. Details as in Table A3.

Acetone (vol%)	Benzoin (mM)	Benzaldehyde (mM)
0	0.70	35.88
10	1.00	81.22
20	1.51	179.14
30	4.08	220.97
40	5.66	280.14
50	16.85	327.0

DMSO (vol%)	Benzoin (mM)	Benzaldehyde (mM)
0	0.7	35.88
10	0.72	79.39
20	1.78	113.07
30	2.78	149.35
40	4.81	182.31
50	8.31	212.32

Ethanol (vol%)	Benzoin (mM)	Benzaldehyde (mM)
0	0.70	35.88
10	2.03	79.60
20	3.74	122.23
30	6.68	187.54
40	7.13	231.14
50	7.95	275.21

2-Propanol (vol%)	Benzoin (mM)	Benzaldehyde (mM)
0	0.70	35.88
10	0.92	73.74
20	0.89	106.62
30	2.39	204.92
40	5.96	262.36
50	9.67	298.36

Table A5: Properties of water-miscible solvents used (Riddick et al, 1986; Khmelnitsky et al., 1991).

Solvent	Log P (-)	Denaturation capacity (-)	Dipole moment (D)	Dielectric constant (-)
DMSO	-1.35	60.3	3.96	47.2
Acetone	-0.24	78.2	2.88	20.7
Ethanol	-0.05	54.4	1.70	24.3
2-propanol	0.07	70.2	1.66	20.2

ACKNOWLEDGEMENTS

I would like to thank so many people for helping me to complete this research!

Special thanks to Prof. Dr. Martina Pohl for offering me this PhD position, for her dedicated supervision, priceless ideas and advice not only concerning scientific topics!

I am very grateful to Dr. Lasse Greiner as my second supervisor, for his valuable contribution, encouragement and discussion on my work.

I thank Prof. Kai Tittmann from the Martin Luther University of Halle-Wittenberg for inviting me and helping me to perform the NMR experiments, for his generous kindness and eagerness. Many thanks also to his friendly students, especially Danilo, for the technical support!

Furthermore, I wish to express my gratitude to all supervisors from BioNoCo: especially Prof. Dr. Jochen Bücks, Prof. Dr. Karl-Erich Jäger, and Dr. A. Spiess for their valuable interpretations and motivating comments. I am extremely thankful to all BioNoCo colleagues, especially Laura Casiraghi for her assistance and motivation during the experiment with $scCO_2$ and Michael Zavrel for his mathematical and engineering contribution and enthusiasm to help me any time.

Also, I would like to thank all my colleagues from the Insitute of Molecular Enzyme Technology, from the office and especially the lab. I cannot list all but I will mention a few: Geral, Lili, Ilona, Sebnem, Melanie, Astrid, the fresh PhDs Uli, Jan and Dörte. Thank you for your friendship, for the nice atmosphere at work, for the lack of complaint, the funny jokes and serious advice, when needed!

I am extremely thankful to my parents for their devotion, patience and love without which all this would not be possible. I love you!

This research was carried out with the financial support of Deutsche Forschungsgemeinschaft, in frame of the research training group “Biocatalysis in non-conventional media” (BioNoCo), which is greatly acknowledged!

LIST OF PAPERS AND PRESENTATIONS

Paper:

Kokova M, Zavrel M, Tittmann K, Spiess AC, Pohl M. 2009. Investigation of the carboligase activity of thiamine diphosphate-dependent enzymes using kinetic modeling and NMR spectroscopy, *J Mol Catal B: Enzym*, 61, 1-2, 73-79.

Poster presentations:

Kokova M, Greiner L, Leitner W, Pohl M. 2007. Characterisation of the influences of non-conventional media on activity and stability of benzaldehyde lyase. 8th International Symposium on Biocatalysis and Biotransformation - Biotrans, Oviedo, Spain.

Kokova M, Tittmann K, Pohl M. 2008. How non-conventional media influence the carboligase activity of thiamine-dependent entymes. 7th International conference on Chemistry and Biology of Thiamine, Wittenberg, Germany.

Kokova M, Tittmann K, Pohl M. 2008. Organic solvents and ionic liquids improve the catalytic performance of thiamine diphosphate- dependent enzymes. 2th International Conference “Biocatalysis in non-conventional media”, Moscow, Russia.

Die vorliegende Dissertation habe ich vollständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in änlicher Form noch bei keiner anderen Institution eingereicht. Ich habe keine erfolglosen Promotionsversuche unternommen.

Aachen, den 8. Oktober, 2009

Mariya Kokova