

A highly stereoselective recombinant alcohol dehydrogenase

from *Pseudomonas fluorescens* DSM50106

Biochemical characterization, substrate specificity, enantioselectivity,
and a new flow-through-polarimetry-based dehydrogenase activity assay

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Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst Moritz Arndt-Universität Greifswald noch an einer anderen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Petra Hildebrandt

Weitenhagen, den 16.11.2005

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Abbreviations

| | |
|----------------------------|--|
| % conversion | Percentage of substrate conversion |
| %ee _p | Percentage enantiomeric excess of the product |
| 2-MeO-AP | 2-Methoxy-acetophenone |
| 3-MeO-AP | 3-Methoxy-acetophenone |
| 4-FI-AP | 4-Fluor-acetophenone |
| ADH <i>L. Kefir</i> | Alcohol dehydrogenase from <i>Lactobacillus Kefir</i> |
| ADH <i>R. erythropolis</i> | Alcohol dehydrogenase from <i>Rhodococcus erythropolis</i> |
| ADH TS | Alcohol dehydrogenase from <i>Thermoanaerobacter spec.</i> |
| APS | Ammonium persulfate |
| BCIP | 5-Bromo-4-chloro-3-indolyl phosphate |
| BSA | Bovine serum albumin |
| BVMO | Baeyer-Villiger monooxygenase |
| DH5 α /pJOE4016 | DH5 α cells transformed with pJOE4016 |
| DMF | Dimethyl formamide |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DNSOA | Dansyloxyamine (<i>N</i> -(5-dimethylamino-1-naphtalenesulphonamido)-3-oxapentane-1,5-dioxyamine) |
| FDH | Formate dehydrogenase |
| DTT | Dithiothreitol |
| GC | Gas chromatography |
| HLADH | Horse liver alcohol dehydrogenase |
| HTS | High-throughput-screening |
| kDa | Relative molecular mass (<i>M_r</i>) in Kilo Dalton |
| LB/Amp ⁺ | Luria-Bertani medium/plates with ampicillin |
| LB-medium/plates | Luria-Bertani medium/plates |
| mM | mmol/l |
| MTS | Medium-throughput screening |
| MW | molecular weight |
| NADH | Reduced nicotinamide adenine dinucleotide |
| NBD-H | 7-Hydrazino-4-nitro-2-oxa-1,3-diazole |
| NBT | Nitro blue tetrazolium chloride |
| OD _{xnm} | Optical density, measured at x nm |

| | |
|--------------|---|
| <i>orf</i> | Open reading frame |
| PB | Sodium phosphate buffer |
| PCR | Polymerase chain reaction |
| PEG 6000 | Polyethylene glycol 6000 |
| Pf- ADH | Alcohol dehydrogenase from <i>Pseudomonas fluorescens</i> |
| pI | Isoelectric point |
| PMS | Phenazine methosulfate |
| RFU | Relative fluorescence unit |
| RT | Retention time |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TEMED | N,N,N',N'-Tetramethyldiamine |
| TLC | Thin layer chromatography |
| TRIS | Tris(hydroxymethyl)aminomethan |
| α -PE | α -Phenyl ethanol |

1. Introduction

1.1. Enzymes in Organic Syntheses

Proteins with catalytic properties are called enzymes. Enzymes accelerate the catalysis of catabolic and anabolic reactions by lowering the energy barrier between substrate and product and stabilizing the transition state, but they cannot change the thermodynamic equilibrium. Enzyme-catalysed reactions can run in both directions. Without catalysis, biochemical reactions would be so slow that life in the known form could not exist.

Already in the 1950ies organic chemists were interested in the remarkable reactions catalysed by oxidosqualene and squalene cyclases and used these enzymes to unravel the mechanisms of different types of reactions (Abe et al. 1993). Enzymes were later on routinely used as catalysts in organic chemistry instead of inorganic catalysts, which are often very expensive and contain often heavy metals known to be hazardous to the environment. The use of enzymes in organic chemistry became more and more interesting since enzymes not only catalyse reactions of their natural substrates, but often also accept synthetic organic compounds. This discovery changed the previously existing dogma that enzymes were absolutely substrate specific and accepted only their natural substrates (Faber 2004).

Advantages of using enzymes for organic syntheses are 1) efficient catalysis often coupled with high stereoselectivity, 2) activity under mild reaction conditions and 3) compatibility with other enzymes in the reaction mixture, which allows reaction cascades in a one-pot-reaction (Faber 2004). As mentioned above, enzymes are not limited to their natural substrates or reaction conditions. There are even examples of enzymes working in organic solvents as long as a limited amount of structural water is present to maintain full enzymatic activity (Zaks and Klivanov 1988; Grunwald et al. 1986; Itoh et al. 1992; Antonini et al. 1981). The log P (partition coefficient) values of organic solvents can be employed to predict the effect of the organic solvent on the catalytic activity and stability of enzymes. The smaller the log P, the more hazardous is the solvent for enzyme structure and stability. For log P values between of 2.0 – 4.0 a prediction is hard to make and applicability has to be experimentally

tested. Organic solvents with high log P values (above 4.0) which are mainly higher alkanes as well as phthalates substituted by long alkyl chains (Peters 2000), are promising candidates to support a prolonged enzyme half life and high enzyme activity.

Enzymes for biotransformation (conversion of artificial substrates with natural enzymes) can be used as whole cell systems, crude protein extracts or partially or highly purified enzymes. The majority of biotransformation is performed with crude (in some cases recombinant) enzyme preparations (containing 1 – 30% of the desired protein), because they are easy to prepare and necessary co-factors are still present. The disadvantage of whole cell systems is that often already relatively low substrate concentrations are cytotoxic and in some cases the offered substrate or products are accepted by more than one enzyme resulting in a decrease in yield and/or stereoselectivity.

Enzymes display three major types of selectivities, which make them so extremely useful for organic synthesis:

- the chemoselectivity, which means that enzymes act on a single type of functional group,
- the regio- and diastereoselectivity that allows the enzyme to distinguish between functional groups that are situated at different regions of the substrate molecule (Sweers and Wong, 1986, Bashir et al. 2003), and
- the enantioselectivity which means that prostereogenic substrates are transformed into the optically active product through a desymmetrisation process. In racemic substrates, the enantiomers react at different rates and afford thereby a kinetic resolution. All natural enzymes are made from L-amino acids and are therefore chiral catalysts.

Enzymes with high stereoselectivity are desirable instruments for the production of enantiopure compounds. For enantiomeric therapeutics it is nowadays provided by law (FDA 1992) to test and prove effects and/or side effects of both enantiomers separately. One of the most well known examples of harmful side effects of mixtures of enantiomers used as drugs in humans is the teratogenic effect of the (*S*)-enantiomer of Thalidomide[®]. The (*R*)-enantiomer of the drug has sedative effects, which has been the reason for the use in humans in the 1960ies. However, since methods for the synthesis of pure enantiomers were

not considered at that time and the harmful effects of the (S)-form was initially not known, the drug was used as racemate also in pregnant women until the teratogenic effect was discovered.

Besides their often excellent stereoselectivity, enzyme-catalysed reactions are often superior to chemical synthesis because they are ecologically beneficial and usually work under less harsh reaction conditions than organic synthesis. This makes enzymes with high stereoselectivity and high conversion very interesting for manufacturers of enantiopure substances.

1.2. Dehydrogenases

Dehydrogenases are enzymes belonging to the group of oxidoreductases (E.C. 1.x.). Within this class, alcohol dehydrogenases (E.C.1.1.1.1, also named keto-reductases) represent an important group of biocatalysts, because they can be used efficiently for syntheses of optically pure alcohols by reduction of the respective prostereogenic ketones (Figure 1).

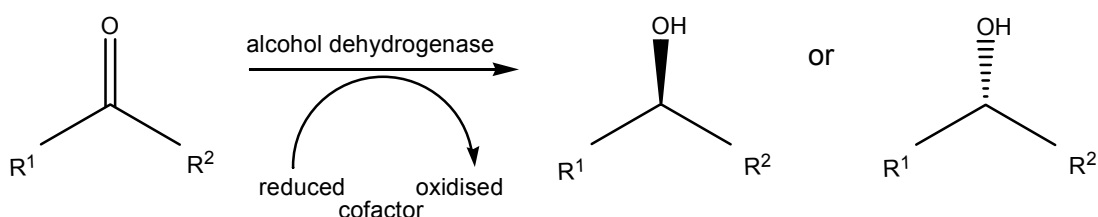


Figure 1. General reduction reactions catalysed by dehydrogenases

A total of 650 oxidoreductases are known from which 80% use NADH as cofactor, 10% the corresponding phosphates. Flavins and pyrroloquinoline quinine are involved more rarely (Peters 2000). The cofactors are still very expensive and their addition to the reaction mixtures in stoichiometric amounts is prohibitively expensive for large-scale reactions. Therefore, only those dehydrogenases, which utilise NADH as cofactor, are yet of industrial importance, because for biocatalysts depending on NADPH much less efficient cofactor recycling systems are available. Since 2004, however Juelich Fine Chemicals (Juelich Fine Chemicals, Karlsruhe, Germany) offers a recombinant alcohol dehydrogenase (E.C.1.1.1.2.) from *Thermoanaerobacter spec.* expressed in *E. coli*, which can be used to recycle NADPH using hydrogen from isopropanol (reference not available, see catalogue from Juelich Fine Chemicals). In

addition to this recycling capacity, this enzyme also exhibits catalytic activity towards aliphatic and aromatic ketones yielding optically pure alcohols in (*S*)-configuration. For the regeneration of the cofactor NADH the formate dehydrogenase (FDH) system from the yeast *Candida boidinii* is widely used (Figure 2). The equilibrium of the reaction catalysed by formate dehydrogenase strongly favours CO₂ formation and reduction of NAD⁺ to NADH (Hummel and Kula 1989; Schütte et al. 1976; Shaked and Whiteside 1980). Sodium formate is a cheap co-substrate and neither formate nor CO₂ influence enzyme or product stability.

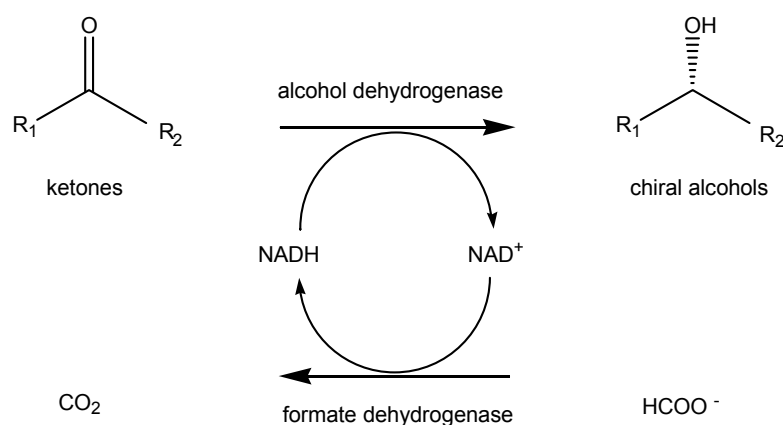


Figure 2. Synthesis of optically pure active compounds using an alcohol dehydrogenase and formate dehydrogenase for regeneration of NADH.

To perform the enantioselective oxidation of racemic alcohols, the NADH regeneration is necessary. Riebel et al. (2003) reported a NADH/NADPH-oxidase from *Lactobacillus sanfranciscensis*, which reduces O₂ to innocuous H₂O and thereby regenerates NADH.

In the literature, several alcohol dehydrogenases have been described (Drauz and Waldmann 1995; Faber 2000; Hummel and Kula 1989; Wong and Whitesides 1994). Amongst these, the most frequently used are from yeast, horse liver and *Thermoanaerobacter* (formerly *Thermonaerobium*) *brockii*, which also differ considerably in their substrate specificity and stereopreference. Only a few ADHs from *Pseudomonas sp.* have been described so far. Shen et al. (1990) reported an enzyme from strain ATCC 49688 that exhibited only limited substrate tolerance, whereas an enzyme from *Pseudomonas sp.* strain PED accepts a wide range of substrates (Bradshaw et al. 1992). Both enzymes are NADH-dependent and generate as reduction products the (*R*)-enantiomers

of the alcohols (Bradshaw et al. 1992). Inoue et al. (2005) isolated an alcohol dehydrogenase from *Leifsonia sp.* S479, which showed broad substrate specificity towards prostereogenic ketones and keto esters and thereby showing mainly anti-Prelog stereopreference. This enzyme was also able to efficiently reproduce NADH when 2-propanol was used as hydrogen donor in the reaction mixture. However, only a few of these enzymes (Stewart 2001) exist in recombinant form and none of the commercially available NADH dependent ADHs is stereoselective for the (*R*)-enantiomer (Kula and Kragl 2000). The *Lactobacillus kefir* ADH is selective for the (*R*)-enantiomer, but it requires NADPH as coenzyme. The same holds true for the ADH from *Lactobacillus brevis* DSM20054 (Hummel & Riebel 2000), which was used for the enzymatic production of chiral hydroxy compounds.

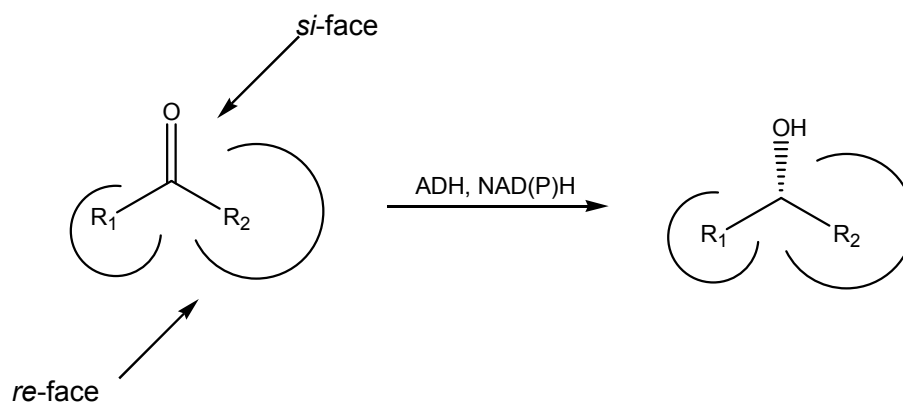


Figure 3. According to the Prelog-rule, the size of the substituents R_1 and R_2 (here $R_1 < R_2$) determines whether the carbonyl of a ketone is attacked by the hydride either from the *re*- or the *si*-face. In the example shown above, the (*S*)-product is formed, if a sequence rule of $R_2 > R_1$ is assumed (from Buchholz, Kasche and Bornscheuer, 2005).

Many of the ADHs were found to obey the Prelog-rule (Figure 3), which is based on the size of the substituent (Prelog 1964) and allows predicting, which enantiomer will be produced, although exceptions have also been described (anti-Prelog).

Tab. 1. Alcohol dehydrogenases from different sources, their selectivities, and cofactor dependence

| ADH | Configuration | Cofactor |
|---------------------------------|---------------------------------|----------|
| Yeast | Prelog | NADH |
| Horse liver | Prelog | NADH |
| <i>Thermoanaerobium brockii</i> | Prelog | NADH |
| <i>Rhodococcus erythropolis</i> | Prelog | NADH |
| <i>Rhodococcus rubber</i> | Prelog | NADH |
| <i>Pseudomonas fluorescens</i> | anti-Prelog/Prelog ^a | NADH |
| <i>Lactobacillus brevis</i> | anti-Prelog | NADPH |
| <i>Lactobacillus kefir</i> | anti-Prelog | NADPH |
| <i>Pseudomonas spec.</i> | anti-Prelog | NADH |

^a configuration depends on the substrate

For the industrial application, the space–time yield of enzyme reactions is important. With the ongoing screening of organisms and metagenomes, directed evolution approaches and the subsequent overexpression of recombinant proteins more enzymes will be found that are suitable for industrial purposes.

This work describes the characterisation of a recombinant alcohol dehydrogenase with a highly stereoselective reaction mechanism that may be suitable for biotechnological applications.

Pseudomonas fluorescens, a water and soil inhabiting bacterial species, is known to oxidise a high number of different organic substances for energy production (Schlegel 1992). In search for enzymes suitable for organic synthesis a genomic library prepared from *Pseudomonas fluorescens* DSM 50106 was sequenced and an *orf* showing 29% identity to a C α -dehydrogenase of *Pseudomonas paucimobilis* (Masai et al. 1993; Hildebrandt et al. 2002) was identified. The sequence showed a high degree of similarity with sequences coding for proteins with unknown functions derived from several genome projects. The corresponding gene *adhF1* encodes a dehydrogenase (Pf-ADH) of 296 amino acids with a calculated molecular mass of 31.997 kDa. Sequencing of the *Ps. fluorescens* genomic library revealed the existence of an esterase and cyclohexanone monooxygenase gene. If both proteins were involved in the energy metabolism of *Ps. fluorescens*, it can be assumed that cyclic alcohols

would be good substrates for the Pf-ADH, yielding the corresponding ketones, which are known to be converted by Baeyer-Villiger-monooxygenases (BVMOs) in a Baeyer-Villiger oxidation (Stewart et al. 1998) to lactones, which, in turn, may be hydrolysed by the esterase. Khalameyzer (1999) showed the high activity of this esterase towards lactones.

2. Aim of the Work

Sequencing the genomic library from *Pseudomonas fluorescens* DSM50106 identified one *orf* with 29% identity to a C α -dehydrogenase from *Pseudomonas paucimobilis* (Masai et al. 1993; Bornscheuer et al. 1998). Dr. Altenbuchner (Hildebrandt et al. 2002) constructed an expression vector containing the gene of the putative dehydrogenase to produce an L-rhamnose inducible expression system in *E.coli*. The C-terminal end of the oxidoreductase gene was fused to six histidine codons of the vector (Stumpp et al. 2000) to facilitate purification of the recombinant enzyme.

For the work on hand, the recombinant *Ps. fluorescens* alcohol dehydrogenase (Pf-ADH) should be functionally expressed in a suitable bacterial host system, purified and characterized in terms of cofactor dependence, kinetic data, solvent stability, substrate specificity and stereoselectivity. To monitor enantioselectivity and conversion rate as well as the substrate specificity of dehydrogenases in medium to high-throughput-systems, a screening assay should be developed.

3. Material and Methods

3.1. Chemicals

Standard chemicals were purchased at analytical grade from Sigma-Aldrich (München, Germany) and Roth (Karlsruhe, Germany) if not stated otherwise.

3.2. Equipment

Autoclave: Astell, Eckold, St.Andreasberg, Germany;

Bacterial incubators: Friocell and Incucell, MMM Medcenter-Einrichtungen, Gräfelfing, Germany;

Laminar flow clean bench: NapFlow 1200-GS, NAPCO, Unterhaching, Germany;

Shaking incubators: Unitron, Infors AG, Bottmingen, Swiss;

Sonicator: Sonoplus Bandelin HD2070, MS 73, Berlin, Germany;

Lyophilisator: Alpha 1-2, Christ, Osterode/Harz, Germany;

Centrifuges: Multifuge 3S-R, Labofuge 400R and Biofuge fresco, Heraeus, Hanau, Germany;

Mikroliter pipettes; Gilson, Bad Camber, Germany;

Magnetic stirrer: IKAMAG[®] safety control, IKA[®] Labortechnik, Staufen, Germany;

Vortexer: Vortex-genie[®], Scientific industry, Bohemia, New York, USA;

pH meter: Microprocessor HI 9321, Hanna Instruments, Kehl am Rhein, Germany;

Thermomixer: Thermomixer comfort, Eppendorf, Hamburg, Germany;

Balance: R180D; Sartorius, Göttingen, Germany;

Gas chromatography: gas chromatograph GC-14A; Chromatopac/Integrator C-R5A or C-R3A; Shimadzu, Duisburg, Germany;

GC columns: (heptakis-(2,3-di-O-acetyl-6-O-*t*.butyldimethylsilyl)- β -cyclodextrin (50% OV in 701) 25 m x 0.25 mm ID or heptakis-(2,6-O-methyl-3-O-pentyl)- β -cyclodextrin, 25 m x 0.25 mm ID, Prof. W. A. König, University of Hamburg, Germany;

UV-Vis-Spectrophotometer: UVmini 1240, Shimadzu, Duisburg, Germany;

Plate reader: Fluostar Galaxy, BMG, Offenburg, Germany;

Power supply: EPS 301, Amersham Pharmacia, Uppsala, Sweden;

Protein gel electrophoresis: Mini-Protean II vertical electrophoresis cell, Biorad, München, Germany;

Electroblotter: Panther semi-dry electro-blotter, Model HEP-1, Peqlab, Erlangen, Germany;

IEF-cell: Protean IEF cell, Biorad, München, Germany;

Flow-through Polarimeter: POLARmonitor, IBZ Meßtechnik, Hannover, Germany;

Liquidhandler: 221xL, Gilson, Bad Camber, Germany;

Peristaltic pump: Minpuls 3, Gilson, Bad Camber, Germany;

Motor driven valve: Besta, Wilhelmsfeld, Germany;

3.3. Plasmid and Host Bacteria

For the expression of the putative alcohol dehydrogenase (Pf-ADH) from *Pseudomonas fluorescens*, the plasmid was constructed based on pJOE3075 (Stumpp et al. 2000). The vector pJOE4016 (Bornscheuer et al. 1998; Hildebrandt et al. 2002) contained in addition to the Pf-ADH gene (sequence in Appendix I) a L-Rhamnose-inducible promoter, the β -lactamase gene for

ampicillin resistance as selection marker and six histidine codons at the C-terminal part of the protein. This histidine-tag should allow easier enzyme purification by metal-affinity chromatography.

As bacterial hosts for protein expression either JM109 or DH5 α (both *E. coli* K12 strains) were used. Both strains were normally cultured in LB-medium at 37°C (1% Bacto-Tryptone, 1% NaCl, 0.5% yeast extract, dissolved in distilled water, autoclaved at 120°C for 20 min, for plasmid harbouring strains the medium was supplemented with 100 μ g/ml ampicillin). The plasmid map in Figure 4 was prepared with the software Clonemanager 5.0 (Scientific & educational software, 1998).

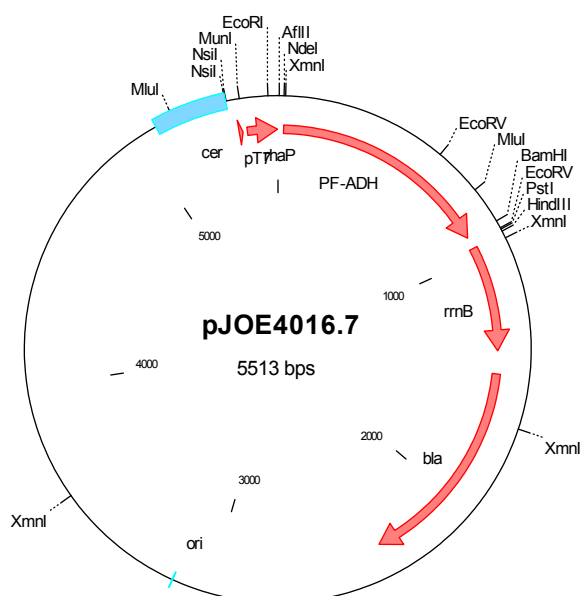


Figure 4. Bacterial plasmid pJOE4016.7 with L-rhamnose promoter (*rhaP*), Pf-ADH gene and β -lactamase gene (*bla*)

3.4. Transformation of Competent *E. coli* Cells

The introduction of heterologous DNA into host bacteria requires competence of the receiving cells that means the capability of bacteria to take up the DNA. One of many standard methods to achieve this transformation competence is the TSS (transfer & storage solution) - method (Chung et al. 1989). Here 50 ml

sterile LB-broth were inoculated 1:100 with a bacterial suspension from an over night grown culture (3-5 ml culture). The cells were harvested when the culture reached the logarithmic phase ($OD_{600nm}=0.4-0.7$; 1 cm path length) by centrifugation for 10 min at 4,000xg at 4°C. The well-dried bacterial pellet was resuspended in 2 ml ice-cold, sterile TSS solution (10% (w/v) PEG 6000, 5% DMSO (v/v), 50 mM $MgSO_4$ or $MgCl_2$, pH 6.5, in LB medium, autoclaved at 120°C for 20 min, stored at 4°C). The resuspended cells were kept on ice for 5 min. To 200 μ l cell suspension 1 μ l plasmid DNA (approx. 50-100 ng DNA) was added, carefully mixed and kept on ice for 20-60 min. After this incubation, the sample was kept for 30-60 sec at 42°C in a thermoshaker or thermostated water bath. According to the original paper by Chung et al. (1989) this heat shock step can also be omitted without significant loss of transformation efficiency, because the plasmid DNA entered the cells already during the incubation step on ice. After adding 800 μ l LB-medium and 18 μ l 20% (w/v) glucose the cells were incubated at 37°C for 1 h to recover before being plated out on LB/Amp⁺ plates (100 μ g/ml sterile Ampicillin were added to the tepid agar solution after autoclaving, 1.5% agar (w/v)). From the cell suspension 25, 50, 100, and 150 μ l were plated out on LB/Amp⁺ plates and the plates were kept in the incubator upside down over night at 37°C.

3.5. Plasmid Preparation

Plasmid preparations were done according to the manufacturer's instructions with the plasmid mini/midi preparation kit from Qiagen.

3.6. Gas Chromatographic Analyses

The chiral gas chromatography (GC) was the method of choice to determine substrate conversion as well as the enantiomeric excess (%ee_p) of the product. All investigated substrates and reduction products were analysed using chiral columns (heptakis-(2,3-di-*O*-acetyl-6-*O*-*t*.butyldimethylsilyl)- β -cyclodextrin (50% OV in 701) 25 m x 0.25 mm ID, Macherey-Nagel, Düren, Germany **or** heptakis-(2,6-*O*-methyl-3-*O*-pentyl)- β -cyclodextrin, 25 m x 0.25 mm ID, Prof. W. A. König, University of Hamburg, Germany; Shimadzu GC14A, equipped with a flame ionization detector, Integrator C5RA or C3RA, Tokyo, Japan). The GC was operated with hydrogen (carrier gas) and nitrogen (make-up gas). The injected sample volume ranged from 0.1-0.4 μ l depending on the compound con-

centration. Further details concerning temperatures and retention times of the substances are reported in section 4.11.

3.7. Cell Cultivation, Harvest and Protein Preparation

In the expression system used, the recombinant protein accumulates in the bacterial cytoplasm. The isolation of the cytoplasmic proteins was done by washing the whole cells and disrupting them by sonication to release the cytoplasm into the washing buffer.

For the large-scale protein preparation, 500 ml LB/Amp⁺ medium were inoculated 1:100 with the cell suspension of an over night culture. The culture was incubated at 37°C on an orbital shaker at 200 rpm until an optical density (OD_{600nm}, 1 cm path length) of 0.5 was reached. The expression of the Pf-ADH was induced by adding 0.2% (v/v) sterile filtered L-rhamnose solution (20% (w/v) L-rhamnose in deionised water) to the culture. After 4-5 h, when the enzyme activity reached its maximum, the cells were harvested by centrifugation (4,000xg, 15 min, 4°C) and washed twice with sodium phosphate buffer (PB, 50 mM, pH 7.5, 4°C, Sambrook et al. 1989). The final bacterial pellet was resuspended in 20 ml PB. Cells were disrupted by sonication on ice for 12 min at 50% pulse and 50% power. Cell debris was removed by centrifugation (4,000xg, 30 min, 4°C) and the supernatant was either directly used for bio-transformations or lyophilised and stored at 4°C in well sealed glass vials.

3.8. Determination of Protein Content (Bradford Assay)

The quantitative estimation of the total protein content of a sample is frequently necessary in cell physiological and biochemical studies. The Bradford assay is a simple, sensitive, and fast method to quantify photometrical the protein content of an unknown sample. It was published by Bradford (1976) and is based on the principle of binding of the dye Coomassie Brilliant Blue G-250 to basic and aromatic amino acids of proteins. After binding to the protein, the peak absorbance of the dye shifts from 465 nm to 595 nm. The linear relationship between absorbance and total protein content over a certain range allows the photometric determination of the protein content.

Protein contents of the samples were determined by using the Bradford method. For the preparation of the stock solution 0.1 g Coomassie Brilliant Blue

G250 were dissolved in 50 ml 50% ethanol. 100 ml 85% phosphoric acid and distilled water were added up to 250 ml, and the solution was stored at 4°C). Bovine serum albumin was used as protein standard (linear range 50-400 µg/ml). The absorption measurements were done in microtiter plates (excitation wavelength 600 nm, 20 flashes/well, 3 cycles; plate reader Fluostar Galaxy) with 300 µl Bradford reagent (1 part Bradford stock solution and 4 parts distilled water, filtered) and 15 µl protein containing sample. Each sample and the standard solutions were measured in triplicate. A sample with PB instead of protein solution served as blank to determine the absorption caused by the Bradford reagent itself. From the standard curve the protein contents of the samples were automatically calculated. Only concentrations within the standard curve values were used for further calculations.

3.9. SDS-PAGE (Laemmli Buffer System)

Polyacrylamide gel electrophoresis separates molecules in complex mixtures according to size and charge. The Laemmli buffer system (Laemmli 1970) is a discontinuous buffer system that incorporates sodium dodecyl sulfate (SDS) in the buffer. In this system, proteins are denatured by heating in buffer containing SDS and a thiol reducing agent such as 2-mercaptoethanol (β -ME). The resultant polypeptides take on a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights. Proteins are separated according to their molecular size, making this system extremely useful for determining molecular mass of proteins.

Stock solutions and buffers:

Ready-to-use 30% acrylamide/N'N'-bis-methylene-acrylamide solution,
37.5:1 mixture (acrylamide/bis solution)

10% SDS (w/v) in deionised water

1.5 M TRIS-HCl (separating gel buffer), adjust to pH 8.8 with 6 M HCl.

0.5 M TRIS-HCl (stacking gel buffer), adjust to pH 6.8 with 6 M HCl

Sample buffer (SDS reducing buffer):

3.55 ml deionised water

1.25 ml 0.5 M TRIS-HCl, pH 6.8

2.5 ml glycerol

2.0 ml 10% (w/v) SDS

0.2 ml 0.5% (w/v) bromophenol blue

0.5 ml β -mercaptoethanol

The sample buffer was aliquoted and kept at -18°C . The samples were diluted between 1:2 and 1:5 with sample buffer and heated to 95°C for 4 min, briefly centrifuged to collect the condensed water and placed on ice until the samples were applied to the gel.

10x Electrode (Running) buffer, pH 8.3:

30.3 g TRIS base

144.0 g glycine

10.0 g SDS

The buffer substances were dissolved and the volume was brought up to 1,000 ml with deionised water. The pH was checked, but not adjusted.

10% (w/v) Ammonium persulfate (APS)

Ammonium persulfate was dissolved in deionised water, aliquoted and stored at -18°C .

Gel formulations (10 ml)

The monomer solution was prepared by mixing all reagents except for TEMED and 10% APS, because they initiate the polymerisation.

| Percent gel [%] | Deionised water [ml] | Acrylamide/Bis solution [ml] | Gel buffer* [ml] | 10% APS [ml] |
|-----------------|----------------------|------------------------------|------------------|--------------|
| 4 | 6.1 | 1.3 | 2.5 | 0.1 |
| 5 | 5.7 | 1.7 | 2.5 | 0.1 |
| 6 | 5.4 | 2.0 | 2.5 | 0.1 |
| 7 | 5.1 | 2.3 | 2.5 | 0.1 |
| 8 | 4.7 | 2.7 | 2.5 | 0.1 |
| 9 | 4.4 | 3.0 | 2.5 | 0.1 |
| 10 | 4.1 | 3.3 | 2.5 | 0.1 |
| 11 | 3.7 | 3.7 | 2.5 | 0.1 |
| 12 | 3.4 | 4.0 | 2.5 | 0.1 |
| 13 | 3.1 | 4.3 | 2.5 | 0.1 |
| 14 | 2.7 | 4.7 | 2.5 | 0.1 |
| 15 | 2.4 | 5.0 | 2.5 | 0.1 |

* Separating gel buffer = 1.5 M TRIS-HCl, pH 8.8, stacking gel buffer = 0.5 M TRIS-HCl, pH 6.8

Immediately prior to pouring the gel(s) the polymerisation was started by adding:

For 10 ml separating gel solution: 50 μ l 10% APS and 5 μ l TEMED

For 10 ml stacking gel solution: 50 μ l 10% APS and 10 μ l TEMED

The solution was swirled gently to start polymerisation and the gels were poured immediately.

All buffers and gel formulations were made according to the user manual of the Protean II vertical electrophoresis cell (Biorad, München, Germany, www.Biorad.com).

The clean and dry glass plates for pouring the gel were assembled as follows: two glass plates were placed on each other with a distance-defining plastic or Teflon spacer between them. Both sides and the bottom of this glass plate sandwich were sealed (see instruction manual of the electrophoresis chamber). Now the separating gel solution (12% bis-acrylamide) was poured between the glass plates. To ensure a smooth gel surface 1-2 ml water-saturated *iso*-butanol was placed on top of the freshly poured gel. After polymerisation, the *iso*-butanol was carefully removed by thorough rinsing with deionised water. Now the stacking gel (normally 4-5% acrylamide/Bis) was poured on top of the separating gel. A comb to form the pockets to take up the samples was inserted between the two glass plates. After completed polymerisation (20-30 min at room temperature) the comb was carefully removed and the gel was assembled to the electrophoresis chamber (see instruction manual). After filling the chamber with 1x running buffer, the sample pockets were rinsed with running buffer and the heated samples were applied to the individual pockets. Low molecular weight protein standards (66 kDa to 6.5 kDa) were applied at least to one lane of each gel to allow the molecular mass determination of the proteins in the unknown samples. Electrophoresis was performed at 120 volts constant for 35-45 min. Due to the SDS coverage all proteins were negatively charged and migrated in the electric field to the anode.

After the bromophenol band had reached the bottom of the gel electrophoresis was stopped and the gel was carefully removed from the glass plates for further manipulations.

3.10. Native PAGE to Determine the Number of Pf-ADH Subunits

The native PAGE was carried out following mainly the SDS-PAGE instructions, but SDS and β -ME were omitted from the gel buffers as well as from the sample buffer. As molecular weight markers, the “nondenatured protein molecular weight marker kit” from SIGMA was employed. To determine the numbers of subunits of the Pf-ADH, native gels with 6-10% acrylamide concentration were prepared and electrophoresis was performed at 50 Volts, 10 mA for 4 h. Enzyme activity staining (see section 3.14) was performed immediately after electrophoresis. Determination of the unknown molecular weight of the native Pf-ADH was done according to the instructions from the nondenatured protein kit from SIGMA (Sigma, Technical Bulletin No. MKR-137). To determine the relative mobility (R_f) of a protein the following formula was used:

$R_f = \text{distance of protein migration} : \text{distance of tracking dye migration}$

$100[\text{Log}(R_f \times 100)]$ values (ordinate) were plotted against the gel concentrations as percent (abscissa) on standard graph paper for each protein. The negative slopes from these graphs were plotted against the known molecular weights of the standards on two cycle log-log paper. The molecular weight of the unknown protein was determined from the graph. As marker proteins served α -lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), and bovine serum albumin (66 kDa).

3.11. Coomassie Staining and Destaining of SDS-Gels

After the separation of the different proteins according to their charge-mass-ratio it was necessary to stain the proteins to detect them. Besides the Coomassie Brilliant Blue staining the silver staining method and fluorescent dyes are available for this purpose. The Coomassie staining is the most widely used staining method, while the silver staining and fluorescent staining methods are mainly used for the detection of spots of low-abundance proteins after 2D-gel electrophoresis. The silver staining method is very sensitive. Down to 10 ng/band protein (tested for BSA) can be detected with this method, while the Coomassie staining will detect protein amounts as low as 300 ng/band.

For Coomassie staining the gel was gently agitated for 30-60 min or over night in the staining solution (0.5 g Coomassie Brilliant Blue R250 was dissolved

in 50 ml methanol or ethanol, 75 ml acetic acid were added and the volume brought to 1000 ml with deionised water. Prior to use, the solution was run through a paper filter to remove non-dissolved dye particles). After staining, the gel was placed for 1-2 h into the destaining solution to remove excess dye (50 ml methanol or ethanol, 75 ml acetic acid, deionised water up to 1,000 ml) until only the protein bands were visible and the gel background became translucent. For the determination of the molecular masses, the NIH Image software for Macintosh computer (<http://rsb.info.nih.gov/nih-image/>) was employed.

3.12. Silver Staining of SDS-Gels

The silver staining of protein bands after gel electrophoresis is a very sensitive but also time-consuming method to detect proteins. During the whole procedure gloves were worn to avoid artificial protein stains from the fingertips. First, the gel was incubated for 1 h (or over night) in 50% (v/v) methanol and 7.5% (v/v) acetic acid, than for 20 min in 5% (v/v) methanol and 7.5% (v/v) acetic acid. After this fixation step the gel is soaked for 30 min in 5% (v/v) freshly prepared aqueous glutaraldehyde solution. Unbound glutaraldehyde was thoroughly removed by washing the gel with multiple volumes of distilled water. The gel was placed into the silver solution for 15 min (0.8 g AgNO₃ dissolved in 80 ml distilled water, 20 ml NaOH and 1.4 ml ammonia) and rinsed afterwards 2-3 times for 5 min with distilled water. The gel was incubated in the developing solution (200 µl 5% (v/v) citric acid, 100 µl 37% (v/v) formaldehyde in 200 ml distilled water) until the staining was completed (5-15 min). After rinsing the gel with water, it was placed in the stop solution (5% (v/v) methanol, 5% (v/v) acetic acid in distilled water) until documented.

3.13. Semi-dry Electro Blotting and Detection of Histidine-Tagged Proteins

Either after electrophoresis the gel was stained or the proteins were electrophoretically transferred (1 h at 1 mA/cm² gel) to a nitrocellulose membrane (Schleicher & Schüll, Germany) using a semi-dry blotting system for further investigations. The blotting buffer was produced according to Qiagen's instructions (25 mM TRIS base, 150 mM glycine, 10% methanol, pH should be 8.3 without adjusting). The detection of histidine-tagged proteins with Ni-NTA-

alkaline phosphatase conjugates was done according to the manufacturer instructions (QIApress detection system, Ni-NTA-alkaline phosphatase conjugates, Qiagen, Hilden, Germany). This detection system is based on the selectivity and affinity of nickel-nitrilotriacetic (Ni-NTA) metal-affinity chromatography matrices for proteins tagged with six consecutive histidine residues. The carbonic anhydrase, often used in protein molecular weight markers (29 kDa), bound to the Ni-NTA AP conjugate served as an intrinsic control. As chromogenic substrate for the conjugated alkaline phosphatase, nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used.

3.14. Enzyme Activity-Staining after SDS-PAGE or Native PAGE

Due to their intrinsic catalytic properties, it is possible to detect enzymes after gel electrophoretic separation by their specific activity towards substrates. For the oxidative activity of dehydrogenases, the PMS/NBT staining system can be applied. During SDS-gel electrophoresis the proteins are denatured and quite possibly not active anymore. By exchanging the negatively charged detergent SDS against a non-ionic detergent at least a partial renaturing and recovery of the enzymatic activity of the proteins is achieved. Therefore, the run SDS-gel is soaked and gently agitated for 1-2 h in renaturing buffer (0.5% Triton-X-100 in 0.1 M TRIS buffer, pH 8.0). The renaturing procedure should not exceed 2 h, because the diffusion of the still unfixed proteins would lead to significant band broadening. To monitor dehydrogenase oxidative activity in the gel tetrazolium salts can be applied. These salts are reduced by hydrogen transferring compounds (NADH, NADPH, etc.) to the corresponding coloured formazan. The gel was incubated in the activity staining solution (7.0 ml 0.1 M TRIS pH 8.0, 400 μ l 10 mM NAD⁺, 400 μ l nitro blue tetrazolium chloride (NBT, 1 mg/ml), 40 μ l phenazine methosulfate (PMS, 1 mg/ml), 200 μ l 500 mM α -phenyl ethanol in isopropanol as dehydrogenase substrate) until the coloured reduction product formazan showed up in the gel. The incubation time was dependent on the activity and the amount of the investigated enzyme and it could take as long as over night for the formazan to form visible signals.

3.15. Isoelectric Focussing

Isoelectric focussing was used to determine the isoelectric point of the recombinant protein. A pH gradient is established by allowing a mixture of low molecular weight organic acids and bases (so-called ampholytes or biolytes) to distribute them in an electric field generated across the focussing strip or gel. When the protein mixture is applied, each protein migrates until it reaches the pH at which all endogen charges add up to zero. This pH represents the isoelectric point (pI) of the protein. Proteins with different isoelectric points are thus distributed differently along the gel strip.

For these experiments focussing gel strips from Biorad were used with an immobilised linear pH-range from 3-10. The gel strip was placed in the focussing tray and the sample (60-100 μ g protein) mixed with rehydration buffer (9 M urea, 2% CHAPS, 50 mM dithiotreitol, 0.2% Biolyte 3-10 (ampholyte)) up to a total volume of 330 μ l was applied. The rehydration was done for 12 h at 20°C, 40 volts and 0.05 mA/gel strip. After the rehydration was finished the instrument automatically started the focussing program (1 h at 200 volts, 1 h at 500 volts, from 500 volts to 10,000 volts within one hour, and 2.5 h at 10,000 volts).

After the isoelectric focussing was finished the activity staining (see section 3.14) of the proteins on the gel strip was performed.

3.16. Biocatalysis

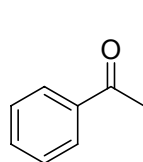
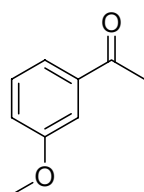
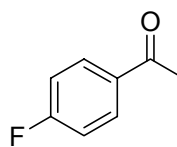
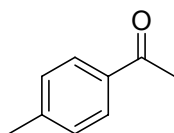
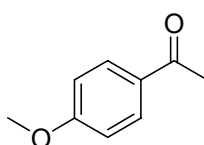
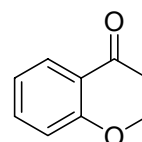
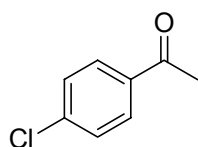
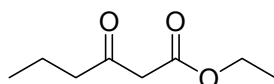
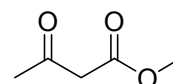
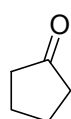
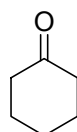
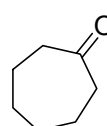
To determine biochemical properties like velocity of reaction, Henri-Michaelis-Menten-constant, substrate specificity, enantioselectivity, temperature and pH-optimum of enzymes, small-scale biotransformations (up to 1 ml in 1.5 ml Eppendorf reaction vials or glass vials) were set up. The reaction products and substrates were extracted from the aqueous reaction mixture and subsequently analysed by chiral gas chromatography.

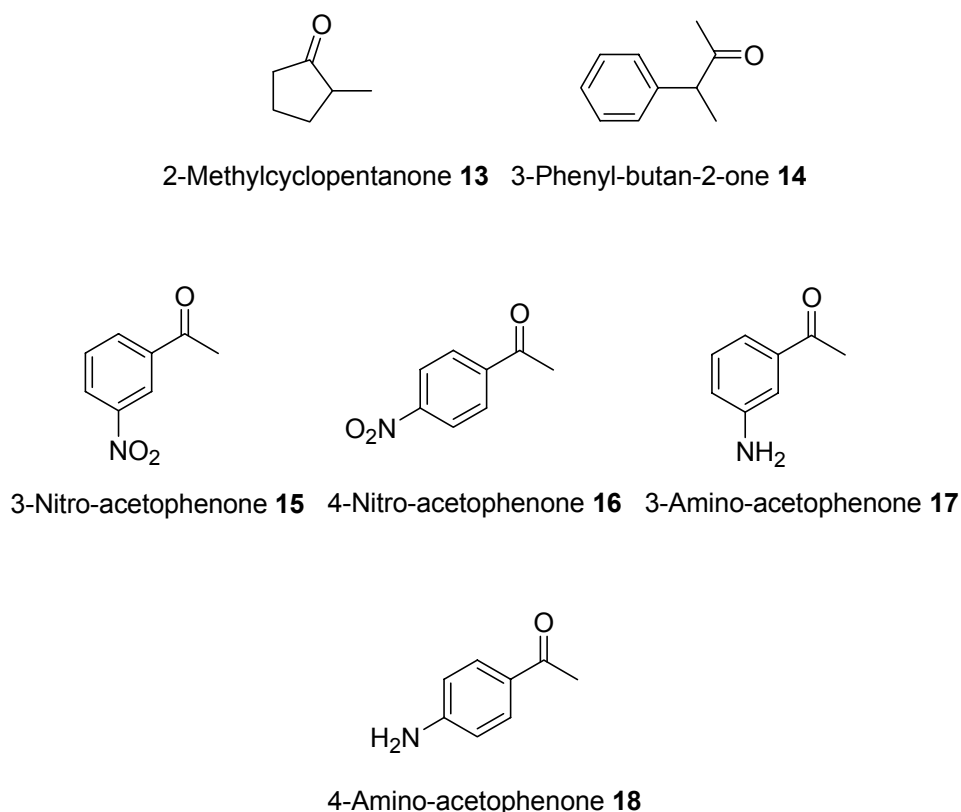
The standard reaction mixture (250-500 μ l) for the reduction of ketones consisted of 12.5-25 μ mol substrate (stock solution 500 mM dissolved in isopropanol), 5 mg/ml protein (crude extract) in TRIS (0.1 M, pH 8.0) and 25-50 μ mol NADH. Experiments with different co-solvents revealed that the addition of NADH could be omitted, when the mixture was supplemented with a total of 20% (v/v) isopropanol. The samples were incubated over night at room tem-

perature. In some experiments reaction conditions different from the above mentioned were used. They are described in detail section 4.8. At the end of the reaction, the products and substrates were extracted twice with one volume chloroform (vortexed for 20 sec, centrifuged at 13.000 rpm for 2 min, Heraeus Biofuge, Hanau, Germany). The bottom organic solvent phases were carefully removed, transferred to a new reaction vial, dried over anhydrous Na_2SO_4 , centrifuged again, transferred to a new vial and analysed immediately by gas chromatography.

Reference substances were available for some of the expected products: enantiopure (*R*)- α -phenyl ethanol, racemic (*R,S*)- α -phenyl ethanol, racemic (*R,S*)-2-methyl cyclopentanol, cyclopentanol, cyclohexanol, and cyclohexanol.

As substrates for the reduction reactions with Pf-ADH the following substances were used:

Acetophenone **1**3-Methoxy-acetophenone **2**4-Fluor-acetophenone **3**4-Methyl-acetophenone **4**4-Methoxy-acetophenone **5**2-Methoxy-acetophenone **6**4-Chlor-acetophenone **7**3-Oxo-hexanoic acid ethylester **8**3-oxo-butyric acid methylester **10**Cyclopentanone **11**Cyclohexanone **12**Cycloheptanone **9**



Scheme 1. Chemical Structures of Pf-ADH substrates used in reduction reactions

A medium scale preparation with 200 mg acetophenone (final volume 33 ml, starting protein concentration 5 mg/ml, 20% isopropanol (v/v), 0.1 M TRIS pH 8.0, room temperature) was performed to test the feasibility of medium scale reaktiokinetic resolution of (*R,S*)- α -phenyl ethanol of Pf-ADH, a typical reaction mixture contained 25 mM racemic α -phenyl ethanol dissolved in isopropanol, 50 mM NAD⁺, 0.1 M TRIS pH 8.0 and 5 mg/ml crude Pf-ADH extract.

3.17. Protein Purification

The recombinant Pf-ADH was expressed with a histidine-tag, which should allow easy purification, by metal-affinity chromatography. For this purpose, the purification with TALON cell thru material (Clontech, BD Biosciences, NJ, USA) was attempted. In this system, metal chelators, which hold Co²⁺ are attached via spacers to sepharose beads. Under physiological conditions histidine binds by sharing electron density of imidazole nitrogen with the electron-deficient orbitals of transition metals. Elution occurs when the imidazole nitrogen is protonated, generating a positively charged ammonium ion, which is repelled by the positively charged metal ion. Alternatively, the bound protein can be competitively eluted by adding imidazole to the elution buffer.

According to the manufacturer's instructions, the appropriate amount of TALON cell thru material was equilibrated with wash buffer (50 mM Na₂HPO₄, 300 mM NaCl, pH 7.4). The Pf-ADH was dissolved in wash buffer, added to the TALON material, and incubated at room temperature for 1 h on an orbital shaker. To remove unbound histidine-tagged protein the TALON material was washed 3 times with wash buffer (20 min at room temperature on a shaker, centrifuged 5 min at 700g). The elution of the enzyme was done by adding two bed volumes of elution buffer (50 mM Na-acetate, 300 mM NaCl, pH 5.0). The elution step was repeated twice. The fractions were collected in reaction vials containing wash buffer pH 7 to elevate the low elution pH. From the biotransformation experiments and the literature it is well known that dehydrogenases often require NADH as cofactor. This NADH dependence can be used to purify enzymes. According to the purification kit manufacturer (Amersham Pharmacia Biotech, Frankfurt/Main, Germany) 5'AMP Sepharose 4B interacts strongly with NAD⁺-dependent enzymes. Elution of bound protein is achieved by a NAD⁺ gradient.

In the first experiment the column (500 µl bed volume) was equilibrated with wash buffer (10 mM PB, 150 mM NaCl, pH 7.4, 1 h binding at room temperature) and the fractions were eluted with a NAD⁺ gradient (2-20 mM in 2 mM steps). These were the conditions recommended by the manufacturer. The experiment was repeated with PB pH 8.0.

Since these two experiments did not result in the desired protein purification, the optimal reaction conditions were determined in small scale experiments (100 µl 5'AMP Sepharose 4B slurry, 100 µl protein solution (10 mg/ml), 200 µl phosphate puffer, 1 h binding time at room temperature, pH 6.0 or pH 8.0, NaCl concentration was varied from 0.05-0.4 M in 0.5M steps). From a liquid protein preparation 50 mg protein were applied twice to the column (pH 6.0 and 0.1 M NaCl, pH 8.0 and 0.3 M NaCl), then the column was washed twice with ten bed volume wash buffer. The elution was done six times with 500 µl of 30 mM NAD⁺.

3.18. Photometric and Fluorimetric Dehydrogenase Activity Tests

The most widely used test to determine reducing dehydrogenase activity is the photometrical NADH-consumption assay. But this assay is not very reliable

when the enzyme of interest is not purified or when medium- to high-throughput screening (MTS or HTS) of crude protein extracts is desired. Besides the NBT/PMS assay, the fluorescent substances NBD-H (Figure 5) and Dansyloxyamine (DNSOA) were tested for their suitability to monitor the conversion of prostereogenic substrates. In the NBT/PMS method the hydrogen transferring substance phenazine methosulfate (PMS) is used to accept the hydrogen from NAD(P)H and transfer it to a tetrazolium salt (NBT), which results in the formation of the coloured formazan (Eisenthal and Danson 2002).

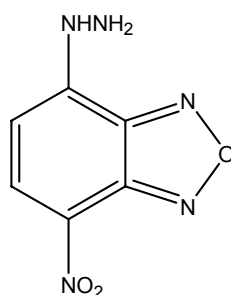


Figure 5. Chemical structure of NBD-H (7-hydrazino-4-nitro-2,1,3-benzoxadiazole) for derivatisation of aldehydes and ketones

For the NBT/PMS assay the same reagents were used as described in section 3.14 for the activity staining. A typical reaction contained 25 μ l substrate solution (7 ml 100 mM TRIS, pH 8.0, 400 μ l 10 mM NAD⁺, 400 μ l NBT (10 mg/ml), 40 μ l PMS (1 mg/ml), 200 μ l 500 mM α -phenyl ethanol) varying protein concentrations (DH5 α or Pf-ADH 50-1000 μ g) and 100 mM TRIS pH 8 up to a total volume of 200 μ l. The absorption maximum of formazan lies at 605 nm (Loba Feinchemie, www.loba.co.at).

The detection of ketones and aldehydes after derivatisation with 7-hydrazino-4-nitro-2-oxa-1,3-diazole (NBD-H) was described by GÜbitz et al. (1984) and Uchiyama et al. (2001). Using the alcohol as a substrate for the Pf-ADH the corresponding ketones should be detectable with the NBD-H. To investigate this, the reaction of ketones and alcohols with the NBD-H were tested under varying conditions for details see section 4.13). In general, NBD-H was dissolved in different solvents (isopropanol, DMSO, DMF, deionised water, methanol/water, 1-propanol) and incubated at room temperature or 50°C with acetophenone, (*R,S*)- α -phenyl ethanol and acetaldehyde as a positive control substance and

the relative fluorescence intensity was measured at 485 nm_{ex} and 520 nm_{em} (thermostated plate reader Galaxy).

Thin layer chromatography (TLC, n-hexane:ethylacetate 5:1, Alugram Sil G/UV₂₅₄, 0.2 mm silica gel with fluorescent indicator, Macherey-Nagel, Düren, Germany) was used to verify the derivatisation of the NBD-H with the reaction partner (after 2 h, 50°C).

Houdier et al. (1999) reported the separation of ketones bound to DNSOA by HPLC (Figure 6). They used DNSOA to detect low concentrations of carbonyls in water. After binding to a compound, fluorescent dyes can shift their emission wavelength. To check whether the DNSOA is useful for the investigations, a test reaction of DNSOA with acetophenone was set up. After incubation at 60°C for 90 min, derivatisation was monitored by TLC (ethyl acetate:hexane 1:5).

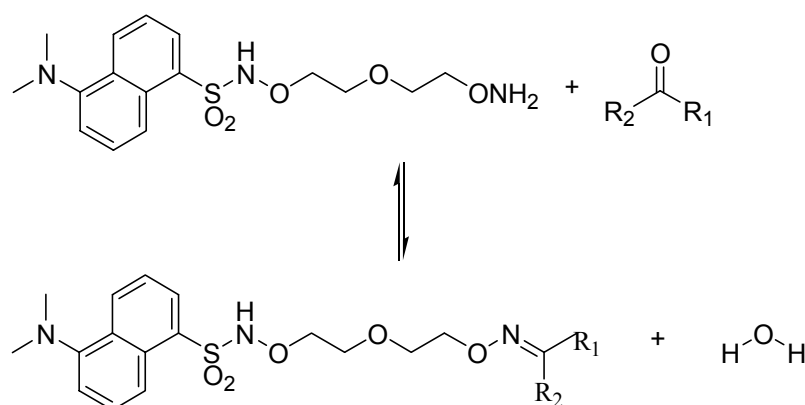


Figure 6. Reaction scheme of DNSOA with ketones to DNSOA oxime ether.

The non-reacted DNSOA was separated from the acetophenone-bound form by silica gel chromatography (ethyl acetate:hexane, 1:5 to remove unbound acetophenone; dichloromethane:methanol, 100:6 to elute the DNSOA-acetophenone). A fluorescence emission and excitation wavelength scan of DNSOA after reaction with acetophenone and non-reacted DNSOA was performed to determine optimal detection wavelengths (Cary Eclipse Fluorescence Spectrophotometer, Varian, Darmstadt, Germany).

3.19. Polarimetric Assay to Determine the Dehydrogenase Activity towards Prostereogenic Substrates

Besides determining NADH consumption or the fluorescence of derivatised compounds, the measurement of the optical rotary dispersion is another possibility to monitor the enantioselective enzymatic activity of oxidoreductases towards optical inactive prostereogenic or racemic substrates that results in optical active compounds. The specific optical rotation $[\alpha]$ of a compound depends on the concentration, the solvent, the temperature, the path length, and the employed wavelength. Air bubbles trapped in the cuvette as well as turbid samples lead to artificial readings due to scattered light and have to be avoided.

To measure the optical rotary dispersion an experimental setup was used which was previously established in our laboratory to determine the activity of a α -amino-acid racemase (Schönfeld and Bornscheuer 2004). The setup consisted of a liquidhandler with keypad (Liquidhandler 221xL, Gilson, Bad Camber, Germany) connected to a peristaltic pump (Minipuls 3, Gilson, Bad Camberg, Germany; peristaltic tube 0.05 cc/m, colour coded blue-white/orange-white, Abimed, Langenfeld, Germany), a motor driven valve (Besta, Wilhelmsfeld, Germany) and a flow-through polarimeter (Polarmonitor, IBZ Meßtechnik, Hannover, Germany) which was equipped with a 300 μ l stainless steel cuvette and a polychromatic light source (Figure 28).

The polychromatic light was polarized and after passing through the sample solution the rotation angle was determined. The sensitivity of the POLARmonitor was set to OR 10 and the average of the displayed readings was set to 20. The xTRAY software controlled the liquidhandler as well as the valve and peristaltic pump. Due to the reaction volume of 2 ml, the samples were prepared and incubated in 24-well-plates (Cellstar, Greiner bio-one, Frickenhausen, Germany). A typical sample contained an oxidoreductase, co-factor (NAD(H)/NADP(H)), prostereogenic ketones or racemic alcohols, buffer, and in reduction mode isopropanol to ensure cofactor recycling. Prior to measurements, the polarimeter was manually set to zero after filling the cuvette with a sample that contained all reaction compounds except the substrate.

The procedure (see the whole program in the Appendix) to run an experiment and record data consisted of the following main steps:

- filling the cuvette for 250 s (approx. 1.5 ml volume sample were pumped at 25 rpm)
- pause for 60 s (to allow the liquid to calm down)
- data recording for 10 s
- next sample is pumped into the cuvette

The remaining 500 μ l sample was removed from the well right after the cuvette was filled to be analysed by GC to test for consistency of data from both analytical techniques. In preparation for GC analysis, samples were extracted twice with one volume chloroform, dried over anhydrous sodium sulfate and analysed by gas chromatography (section 3.6).

4. Results and Discussion

4.1. Protein Preparation

Biotransformations can be performed in whole cell systems or with protein preparations of different purity. Whole cell systems are easier to handle and require less work, but since most substrates are toxic to the cells at higher concentrations, the resulting yield may be lower. Another problem is that purification of the products is generally more demanding. Moreover, the enantioselectivity may be lower since the cells may express more than one enzyme that accepts the substrate in question. The use of protein preparations with overexpressed recombinant enzyme or (partially) purified proteins can circumvent these problems.

As host organisms JM109 and DH5 α , both are *E.coli* K12 laboratory strains, were used. Due to mutations in their recA1 genes, a good insert stability is realised, because they are unable to perform homologous recombination. The Pf-ADH coding *adhF1* gene is cloned into the pJOE3075. This plasmid contains in addition to the PF-ADH coding gene, the β -lactamase gene for ampicillin resistance and it is regulated by a L-rhamnose-inducible promoter. For easier purification of the recombinant protein, a tail of six histidine residues is attached to the C-terminal part.

To determine the optimal time for harvesting the transformed bacterial cells to obtain the highest activity of the recombinant Pf-ADH, a 100 ml culture of DH5 α /pJOE4016 was induced with L-rhamnose at OD_{600nm} = 0.5 and 2 ml samples were taken at 2 h, 4 h, and 22 h after induction. The cells were washed, resuspended in 1 ml PB and disrupted by ultrasound treatment (1 min, 50% pulse, 50% power). The protein content of the supernatants was determined by the Bradford assay with BSA as protein standard. For the determination of the enzyme activity by GC analysis 800 μ l supernatant were mixed with 100 μ l isopropanol and 50 μ mol acetophenone for biocatalysis and incubated overnight at room temperature.

The highest enzyme activity in the crude protein preparation was observed when the cultures were harvested 4 h after induction. Therefore, the preparation of cell lysates was routinely done after this 4 h of induction. The protein prepa-

rations were usually frozen immediately after preparation at $-80\text{ }^{\circ}\text{C}$ and afterwards freeze-dried overnight. A typical protein preparation showed a protein content between $300\text{-}600\text{ }\mu\text{g}/\text{mg}_{\text{lyophilisate}}$. For control experiments plasmid lacking DH5 α cells were cultivated in LB-medium, harvested, and freeze-dried and stored at $-20\text{ }^{\circ}\text{C}$ prior to use.

4.2. Verification of Protein Expression and Molecular Weight

Gene induction, overexpression of the recombinant protein, and the calculated molecular weight of the over expressed protein were confirmed by SDS-PAGE (Figure 7). The presence of the histidine-tag was substantiated by Ni-NTA AP conjugate (Figure 8) after semi-dry electro-blotting of the separated proteins to a sheet of nitrocellulose. The calculated molecular weight from Pf-ADH of 32 kDa and the induction of expression by the L-rhamnose promoter could thus be confirmed. In Figure 7 in lanes 6 and 7 the proteins from the pellet fraction were separated. Here, 10 μl pellet fraction which contained mainly non-soluble proteins were resuspended in 5 μl reducing sample buffer containing SDS and β -ME and heated for 5 min at 95°C . The prominent signal (marked by frame) is caused by insoluble Pf-ADH, which accumulated in the microbial cytoplasm in form of inclusion bodies.

However, besides depositing the majority of recombinant enzyme molecules in inclusion bodies, the bacteria produced also significant amounts of soluble Pf-ADH that could be detected in the cytosolic fraction of bacterial lysate (Figure 7 lane 5). As determined by "NIH image" densitometric software the amount of soluble recombinant protein was approximately 20% of the total protein content. The production of inclusion bodies is an often encountered problem, when proteins are heavily overexpressed in bacterial systems (Hoffmann et al. 2001).

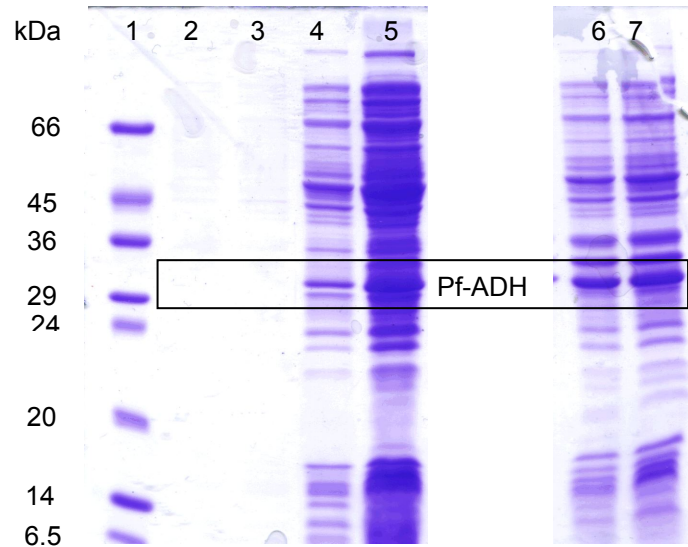


Figure 7. Coomassie-stained 12% SDS-gel of cytosolic fractions of bacterial lysates: before induction of Pf-ADH gene expression (lanes 2 and 3), 2 h after induction (lane 4), 4 h after induction (lane 5) or inclusion bodies from the pellet fraction 4 h after induction (lanes 6 and 7), respectively. Low molecular weight marker from SIGMA in lane 1.

The successful detection of the histidine-tagged dehydrogenase by Ni-NTA AP conjugate confirmed the molecular weight of 32 kDa and the functional expression of the histidine-tag (Figure 8).

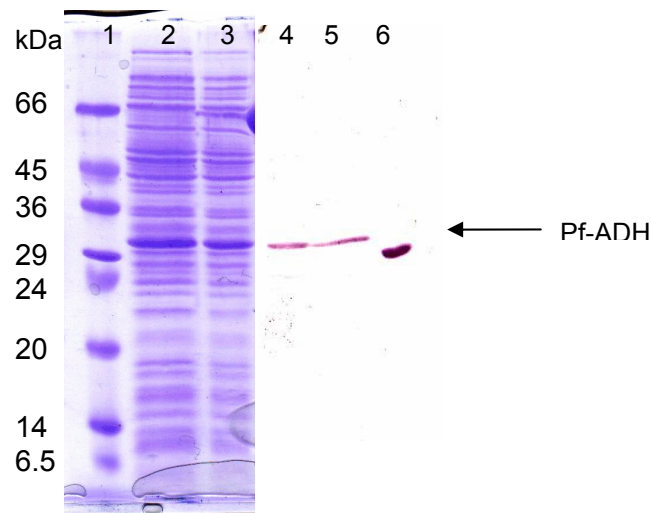


Figure 8. Coomassie-stained 12% SDS-gel molecular weight marker (lane 1), 10 µg crude Pf-ADH extract after 4 h of induction (lane 2), 5 µg crude Pf-ADH extract after 4 h of induction (lane 3), electro blot of SDS-gel treated with Ni-NTA-alkaline phosphatase conjugate to detect histidine-tagged Pf-ADH (lanes 4 and 5), molecular weight marker carbonic anhydrase signal (lane 6).

The strong colour in lane 6 (molecular weight marker) is caused by the carbonic anhydrase, a metalloenzyme with one zinc ion per protein molecule, which forms a complex with Ni-NTA-conjugates (Tanis et al. 1974) and thus served as internal control for the detection system, as well as molecular weight marker at 29 kDa.

4.3. Determination of the Number of Pf-ADH Subunits

The crude protein extract was submitted to native gel electrophoresis and Pf-ADH was localised by enzyme activity staining employing the NBT/PMS system with racemic α -phenyl ethanol as the substrate. Only one major protein band became visible after this treatment (Figure 9).

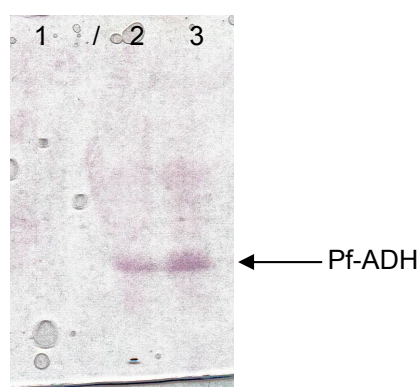


Figure 9. Activity staining with the PMS/NBT system after native PAGE of Pf-ADH crude extract and control extract, which was lacking the Pf-ADH gene. Lane 1: DH5 α cell lysate (10 μ g protein), lane 2 and 3: crude Pf-ADH cell lysate (10 μ g and 20 μ g respectively).

The molecular weight of the nondenatured Pf-ADH was calculated to \sim 58 kDa, which suggests that the natural enzyme is present as a homodimer. For the *Azoarcus sp.* secondary alcohol dehydrogenase, Knimeyer & Heider (2001) reported that the native enzyme consists of two subunits of 25.5 kDa each. Recently, Kosjek et al. (2004) described also a homodimeric chemotolerant alcohol dehydrogenase from *Rhodococcus rubber*. Together, these results indicate that bacterial ADHs occur as homodimers. However, there are also reports about monomeric or tetrameric bacterial dehydrogenases (Olofsson et al. 2005). So dimer-formation observed for Pf-ADH is not the rule in bacterial dehydrogenases.

4.4. Isoelectric Focussing to Reveal the Isoelectric Point (pI) of Pf-ADH

The enzyme activity staining using the NBT/PMS method (see section 3.14) subsequent to the isoelectric focussing revealed one prominent band on the focussing strip. The pI of this protein was calculated from the IEF to be pH 5.7. The theoretical pI, which was calculated with the software Expasy pI/MW tool ([www. Expasy.com](http://www.Expasy.com)), was 9.08. Omitting the six histidine residues at the C-terminus for theoretical calculations made no difference. For a soluble cytoplasmic protein like the Pf-ADH this theoretical pI seems to be unreasonable high. A literature search in PubMed-listed journals for the isoelectric point of microbial ADH revealed that for the majority of ADHs an isoelectric point of pH 4-7 is common (Yoon et al. 2002; Larroy et al. 2002; Dausmann et al. 1997; van Irsel et al. 1997; Uttaro and Opperdoes 1997). With a pI of 9.1 the ADH from *Ralstonia eutropha* was an exception of this rule (Zarnt et al. 1997). Human and rabbit liver ADHs showed pIs in the range of 7 to 9 (Martinovic et al. 2000; Keung et al. 1995; Ditlow et al. 1984).

Although no negative control with DH5 α lyophilisate was carried out, it can be assumed that this band represents the Pf-ADH (Figure 10), because in all enzyme activity stainings performed in 1D-gels with racemic α -phenyl ethanol as substrate only a single protein band was present. Since in preparation for the IEF proteins were denatured in urea/thiourea solutions, this result shows that the dehydrogenase is catalytically active as a monomer or that dimerisation of Pf-ADH monomers is possible even after washing out the denaturing reagents from the gel matrix during the activity staining procedure.



Figure 10. Isoelectric focussing gel strip (linear pH 3-10) after enzyme activity staining using racemic α -phenyl ethanol as substrate.

4.5. Kinetic Data

To determine the maximum reaction velocity (V_{\max}) and the Henri-Michaelis-Menten-constant (K_m), to describe the enzyme affinity to its substrate, reaction mixtures were set up (1.5-25 mM acetophenone, 5 mg/ml protein extract, final volume 500 μ l, 0.1 M TRIS pH 8.0, 20% isopropanol (v/v)) and extracted after one hour incubation with two volumes of chloroform. GC analysis was per-

formed as described in section 3.6. From the Eadie-Hofstee-plot, V_{\max} was calculated to $6 \text{ nmol min}^{-1} \text{ mg}^{-1}$, and the K_m -value was 5.9 mM . The specific activity for acetophenone was determined to $1.1 \text{ nmol min}^{-1} \text{ mg}^{-1}_{\text{prot}}$.

From a practical point of view, a V_{\max} of $6 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein is not sufficient to use this enzyme effectively in large-scale biotechnological procedures. However, the enzyme is highly (*R*)-enantioselective and therefore quite interesting for research purposes in terms of catalysing stereospecific reactions (desymmetrisation of prostereogenic ketones and kinetic resolution of racemic alcohols). For industrial use, it would be necessary to enhance the enzyme activity by either directed evolution, possible without knowledge about the enzyme three-dimensional structure, or by site-directed mutagenesis. This approach requires knowledge about the enzyme structure.

4.6. Cofactor Dependence

From the 3500 known enzymes about 70% are cofactor-dependent. A total of 650 oxidoreductases are known from which 80% use NADH as cofactor (Figure 11), 10% the corresponding phosphate. Flavins and pyrroloquinoline quinine are required as cofactors in some enzymes (Peters 2000).

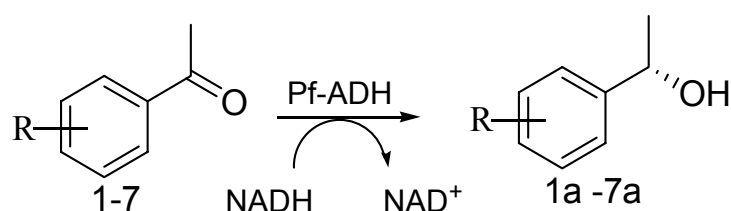


Figure 11. Principle of the Pf-ADH catalysed reduction of arylaliphatic ketones 1-7 (see Scheme 1 also for the definition of R to the corresponding alcohols).

To determine the requirement of a cofactor for the activity of the Pf-ADH, bacterial lysates were dialysed against PB to remove the cofactor and the dialysed protein solution was checked for catalytic activity. For control experiments, non-dialysed protein preparations and dialysed protein preparations with added exogenous NADH were used.

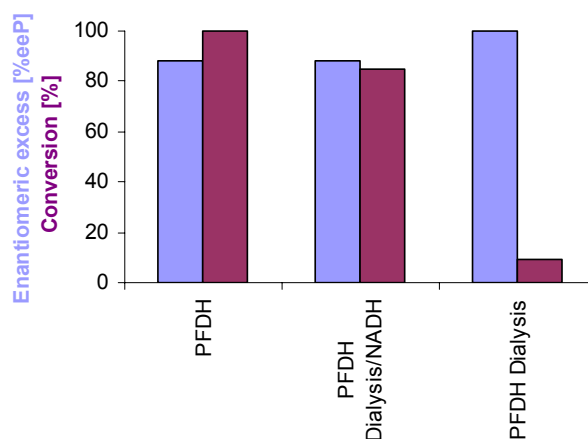


Figure 12. Enantiomeric excess (blue bars) and enzyme activity (purple bars) of Pf-ADH-mediated reactions depending upon NADH as cofactor. PFDH – fresh bacterial lysates, substrate and endogenous NADH (control and set as 100% reference value), PFDH/Dialysis/NADH - dialysed bacterial lysates, substrate and exogenous NADH, PFDH/Dialysis – dialysed bacterial lysates, substrates without NADH.

Total activity of the enzyme (Figure 12), however, is clearly dependent on NADH as cofactor since the absence of NADH reduced enzyme activity to approximately 6% of the activity observed in reactions using dialysed bacterial lysates. The almost complete restoring of activity observed in reactions using dialysed bacterial lysates in the presence of exogenous NADH clearly indicates that the Pf-ADH is a NADH-dependent enzyme.

4.7. Co-Solvent and NADH-Recycling

In small scale biotransformations (final volume 500 μ l, 5 mg/ml protein extract, 50 μ mol NADH, 50 mM TRIS pH 7.5, 25 μ mol substrate in different co-solvents, incubation for 24 h at room temperature) the influence of the solvents, in which the water-immiscible substrate acetophenone was dissolved and added to the reaction mixture, was investigated. The starting co-solvent concentration was 10% (v/v). As co-solvents the water-miscible DMSO, ethanol, isopropanol and acetone were tested. The GC samples were prepared as described in section 3.6. For the GC analysis the injector temperature was set to 200°C, detector temperature was 170°C and column temperature was kept isothermal at 120°C. In the original GC setup, retention times for acetophenone and the α -phenyl ethanol enantiomers were 4.9 min and 8.8/9.3 min, respectively. After the GC reconfiguration (gas flows/split ratio, column change)

retention times changed to 1.54 min for acetophenone and 2.7/2.9 min for (*R*)- α -phenyl ethanol/(*S*)- α -phenyl ethanol.

The highest conversion of acetophenone (66%) was accomplished when isopropanol was used as co-solvent (Figure 13).

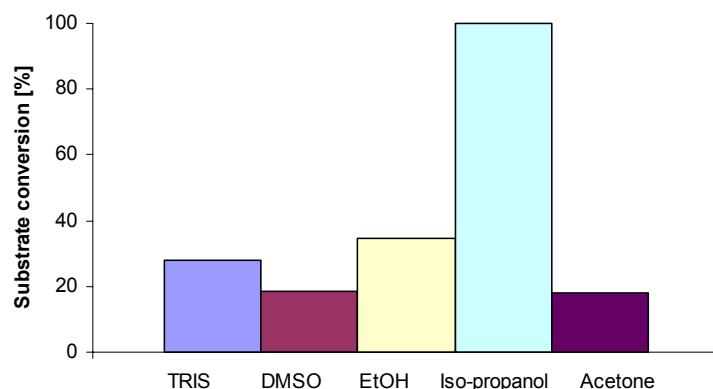


Figure 13. Influence of different co-solvents on the conversion of acetophenone to α -phenyl ethanol by Pf-ADH. The isopropanol containing sample was set as 100% reference value.

For samples with DMSO, ethanol and acetone as co-solvents the substrate conversion was significantly lower and reached only 12-22% compared to isopropanol. Acetone, as we will see later, is a by-product of NADH-recycling via isopropanol and it will shift the reaction equilibrium to the oxidized state of the substrate (Figure 14).

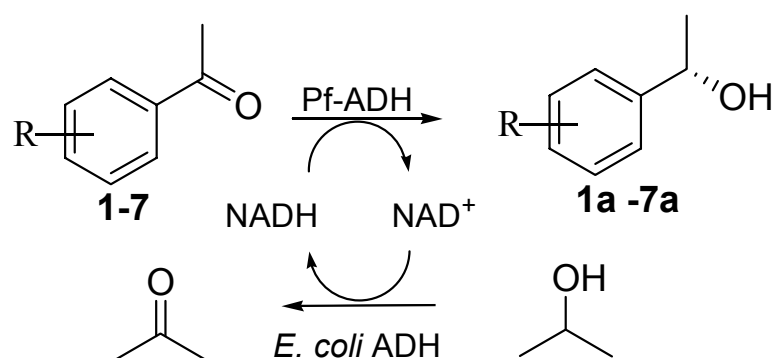


Figure 14. Principle of the Pf-ADH catalysed reduction of arylaliphatic ketones 1-7 in combination with cofactor-recycling catalysed by a dehydrogenase (DH) present in the crude cell extract from *E. coli*.

It was necessary to determine in this monophasic aqueous-organic solution the optimal isopropanol concentration that allowed the highest substrate con-

version. Reaction mixtures with 5-50% isopropanol were set up, incubated and analysed as described before.

As shown in Figure 15, the highest substrate conversion was reached when 20% isopropanol were added to the reaction mixture. Adding 20% isopropanol may support conversion, since a greater part of the substrate will be dissolved and is therefore more easily available to the active site of the enzyme.

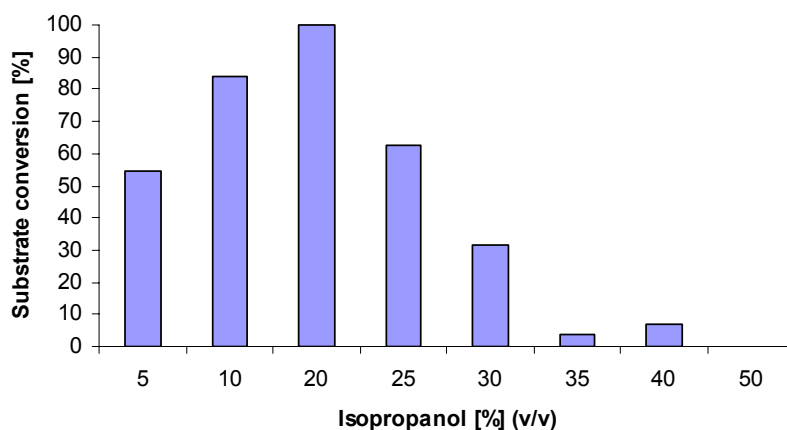


Figure 15. Influence of different isopropanol concentrations on substrate conversion (acetophenone) in the presence of added NADH. The 20% isopropanol value (conversion 86%) was used as 100% reference value.

The addition of organic solvents in monophasic aqueous-organic solvent solutions is usually not problematic up to ~10% of the total volume. Above this threshold, the essential bound water is stripped from the enzyme's surface often resulting in enzyme deactivation (Faber 2004). Water in biological systems or enzymatic reactions exists in two forms: the majority of the water is bulk water, the solvent itself, whereas the water tightly bound to the enzyme is referred to as the bound or structural water, which serves a structural component that stabilises the protein conformation of the enzyme. Kosjek et al. (2003) described a chemotolerant alcohol dehydrogenase from *Rhodococcus ruber* DSM44541 that withstand elevated concentrations of organic solvents, such as acetone (up to 50%) and 2-propanol (up to 80%) and showed in both cases a relative initial activity of nearly 100%.

Some co-solvents may not only affect protein conformation or substrate availability, but may themselves take part in the reaction. As mentioned above, acetone may be a reaction product of dehydrogenase reactions and, therefore,

can cause product inhibition or be involved in the reverse reaction when added in high concentrations to the reaction mixture. Moreover, the reaction equilibrium may in one case be shifted to the oxidated, in another case to the reduced products, depending on the chosen reaction conditions.

To test whether isopropanol, which may be degraded and used as carbon source by enzyme cascades in bacterial lysates, may not only serve as a co-solvent but also as a substrate for the regeneration of NADH, bacterial lysates containing recombinant Pf-ADH were incubated with different volume fractions of isopropanol without the addition of exogenous NADH.

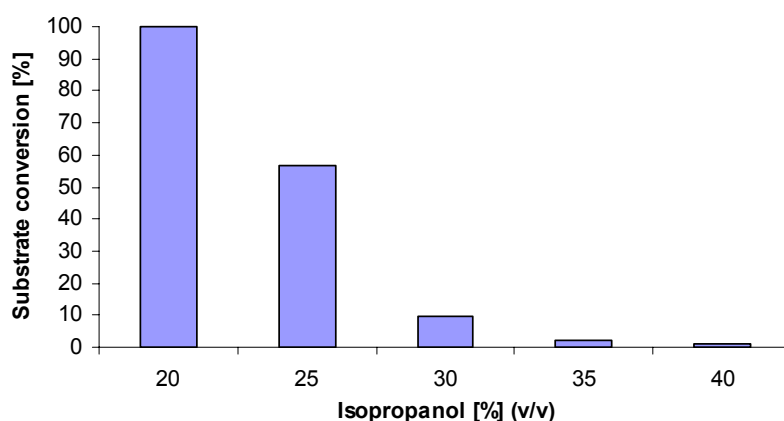


Figure 16. Influence of different isopropanol concentrations on substrate conversion (acetophenone) without additional NADH. The 20% (v/v) isopropanol value was used as 100% reference.

As shown in Figure 16, addition of 20% isopropanol (v/v) resulted in maximum conversion in the absence of exogenous cofactor. Bacterial lysates contained obviously sufficient NAD^+/NADH to support dehydrogenase reactions. Supposedly, recycling of NADH occurred by oxidation of isopropanol (Figure 16). Isopropanol concentrations above 20% attenuated enzyme activity probably due to the removal of structural water. Control experiments using a DH5 α *E. coli* strain lacking the Pf-ADH gene suggested that NADH-recycling is catalysed by a constitutively active *E. coli* alcohol dehydrogenase. This result was obtained with ethyl pyruvate as the substrate, which was according to GC analysis completely metabolised by the DH5 α control preparation.

In summary, excess isopropanol, which is a very inexpensive co-solvent in biotechnological processes, may serve also as substrate for NADH-regenera-

tion and its presence pushes the equilibrium towards the reduction of substrates (Nakamura et al. 2003). This is a favourable situation, since it makes addition of very expensive exogenous co-factors to the dehydrogenase reaction mixtures obsolete.

4.8. pH- and Temperature Optimum and Enzyme Stability

To determine the pH- and temperature-optimum of the Pf-ADH, small scale biotransformations with acetophenone as model substrate (250/500 μ l reaction mixture containing 10/50 mg/ml Pf-ADH crude protein extract, 6.25 /12.5 μ mol acetophenone, 20% isopropanol, 0.1 M TRIS) were incubated overnight at room temperature and analysed by GC. The GC was operated at the following conditions: injection temperature was set to 200°C, the isothermal column temperature was 120°C, and the detector was heated to 170°C. Under these conditions the acetophenone was detected at a retention time of 1.55 min, the (*R*)- α -phenyl ethanol at 2.7 min and the (*S*)- α -phenyl ethanol at 2.9 min (Figure 17).

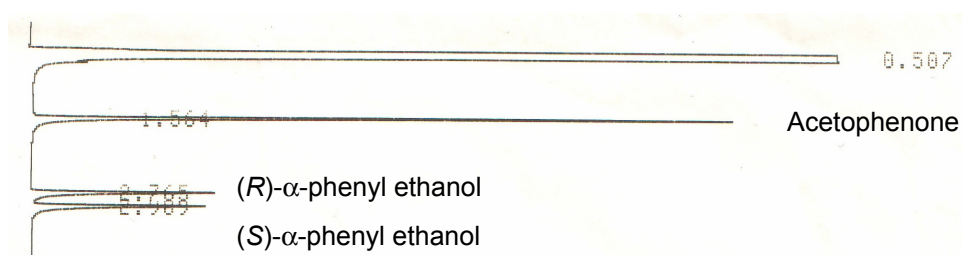


Figure 17. Example chromatogram of the GC-separation of acetophenone and (*R,S*)- α -1-phenyl ethanol. The elution order was defined using the enantiopure (*R*)- α -1-phenyl ethanol as reference.

The pH optimum of recombinant Pf-ADH was determined in TRIS buffered solutions setting the pH to values between 6 and 10. The highest reductive substrate conversion (Figure 18) was obtained using buffer pH 8.0, while the enantiomeric excess (%*ee*_P) was stable over the range of pH 7-9, but it dropped significantly when the pH was lowered to pH 6 or raised to pH 10.

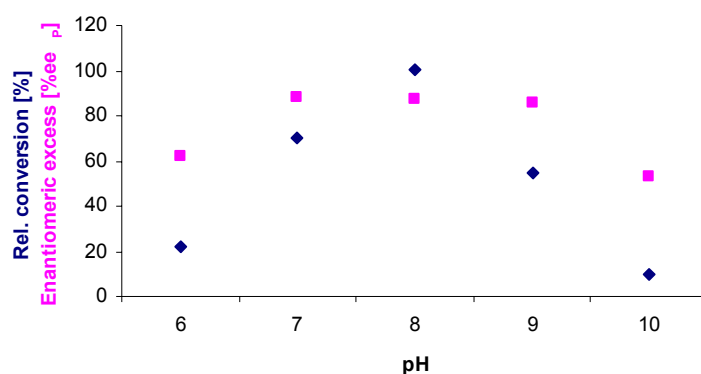


Figure 18. Determination of the pH-optimum of recombinant Pf-ADH with acetophenone as substrate in small scale biotransformations [n=3], the conversion values at pH 8 were set to 100% for calculations.

Since other dehydrogenases were reported to show different pH optima in forward and reverse reaction directions (e.g. pH 8-9 for substrate oxidation and pH 7 for substrate reduction by HLADH, Peters 2000), the oxidation of racemic α -phenyl ethanol by recombinant Pf-ADH was investigated at pH 8.0. Under these conditions, enzyme activity and kinetic resolution were high (selective for the (*R*)-enantiomer) indicating that pH 8.0 seems to be suitable for use of Pf-ADH in oxidation and reduction reactions. However, since other substrates or different reaction conditions may affect the pH-dependence of reactions (Sund and Theorell 1963), an overall pH-optimum cannot be defined and has to be experimentally determined for each individual reaction system.

Another important factor influencing catalytic activity and yield of an enzymatic reaction is the reaction temperature. It was expected that an enzyme from *Pseudomonas fluorescens*, which is a soil and water dwelling bacterium living in temperate as well as cold climate zones should have a temperature optimum between 10 and 20°C. Experiments using recombinant Pf-ADH and acetophenone as substrate showed (Figure 19) that his enzyme has a broad temperature-optimum range with a maximum between 15 and 20°C. The enzyme activity is less affected by lower than by higher temperature (25°C). Stereoselectivity of the enzyme however is not affected by temperature since enantiomeric excess [%ee_p] and stereopreference for the tested substrates were not affected by lowering or increasing the temperature. This is in contrast to a few reports in the literature that the reaction temperature is one of the

parameters affecting the enantioselectivity of other bacterial ADHs (Pham et al. 1989, Zheng et al. 1994, Tripp et al. 1998, Heiss et al. 2001).

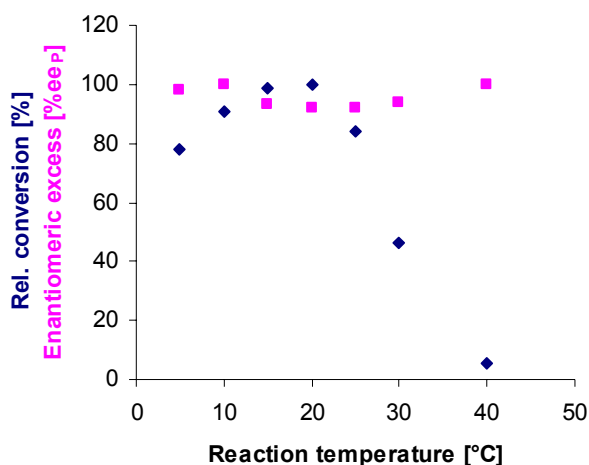


Figure 19. Determination of the temperature-optimum of Pf-ADH with acetophenone as substrate in small scale biotransformations [n=3], pink squares represent the relative conversion [%], the blue squares the enantiomeric excess [%ee_P].

The optimal reaction temperature was revealed also for the acetophenone derivatives 2- and 3-methoxy acetophenone, 4-fluoro acetophenone, and for cyclohexanone. The reaction mixtures contained 50 mg/ml Pf-ADH crude protein extract, 6.25 μmol substrate, 0.1 M TRIS pH 8.0 in a final reaction volume of 250 μl.

Tab 2. Analysis of substrates and products of Pf-ADH reaction

| Substrate | GC temperature program | Retention times ^a [min] substrate/product ^b |
|-----------------|-------------------------------|---|
| 6 /2-MeO | 120 °C 2 min/10 °C/min 150 °C | 3.7/4.9/4.98 |
| 3 /4-F | 120 °C 2 min/10 °C/min 150 °C | 1.58/3.03/3.28 |
| 2 /3-MeO | 120 °C 2 min/10 °C/min 150 °C | 3.95/5.59/5.85 |
| 12 | 90 °C 1 min, 5 °C/min 150 °C | 1.43/2.12 |

^a Note: the retention times are no fix values, because they depend on the actual configuration of the GC. ^b for chiral compounds **2**, **3**, and **6** the retention times for both enantiomers are given.

For all tested substances the optimal reaction temperature to reach the best substrate conversion ranged from 15-20°C (Tab. 3). Cyclohexanone was reduced with the highest activity and in a quantitative manner over the broad temperature range from 5-25 °C. 4-Fluoro acetophenone and 3-methoxy acetophenone were converted with high activity (83% to 93% conversion) and

good stereoselectivity (88 to 93%ee_P) in the temperature range from 10-20°C. Temperatures above 30°C lead to a significant decrease of activity (27-59% conversion) towards all tested substrates. For 2-methoxy acetophenone 40% was the highest conversion reached at 15°C reaction temperature.

The GC settings (Tab 2) for the analyses of the reaction products were: injection temperature 200°C and detector temperature 170°C. Enantiopure reference substance (*R*)- α -phenyl ethanol) was used to assign peaks/retention times from the GC-analyses to compounds in the reaction mixture. For the enantiomers of the other acetophenone derivatives the same elution order was assumed. As for the experiments with acetophenone as substrate no change in enantioselectivity due to temperature variation was observed.

Tab. 3. Desymmetrisation of acetophenone derivatives and reduction of cyclohexanone at different temperatures (% conversion: fraction of product in% of the sum of substrate and product, 20 h).

| T ^a [°C] | 6 E ^b [%ee _P] | Conversion [%] | 3 E ^b [%ee _P] | Conversion [%] | 2 E ^b [%ee _P] | Conversion [%] | 12 Conversion [%] |
|------------------------|---|-------------------|---|-------------------|---|-------------------|--------------------------------|
| 5 | 100 | 25 | 94 | 74 | 81 | 91 | >99 |
| 10 | 100 | 27 | 93 | 86 | 88 | 83 | >99 |
| 15 | 100 | 40 | 91 | 93 | 91 | 93 | >99 |
| 20 | 100 | 31 | 91 | 91 | 92 | 89 | >99 |
| 25 | 100 | 42 ^c | 92 | 60 | 99 | 84 | >99 |
| 30 | 100 | 24 | 93 | 27 | 94 | 46 | 59 |
| 40 | / | 0 | 100 | 3 | 100 | 8 | 13 |

^a Temperature, ^b Enantiomeric excess, ^c This value is probably due to a sample error.

The stability of enzymatic activity of Pf-ADH crude protein extracts was assessed by incubating the dissolved enzyme for 2 h prior to the addition of substrate at different temperatures (5 mg/ml Pf-ADH crude protein extract, 0.1 M TRIS pH 8.0, 20% isopropanol (v/v), 12.5 μ mol acetophenone, final volume 500 μ l). In Figure 20, the decrease in enzymatic activity after pre-incubation is shown. A decrease of 20% in enzymatic activity was observed already after 2 h at 20°C. Higher temperatures led to an even greater loss in activity. Pre-incubation at 40 °C led to a 90% loss of activity within 2 h in comparison to the non-treated sample.

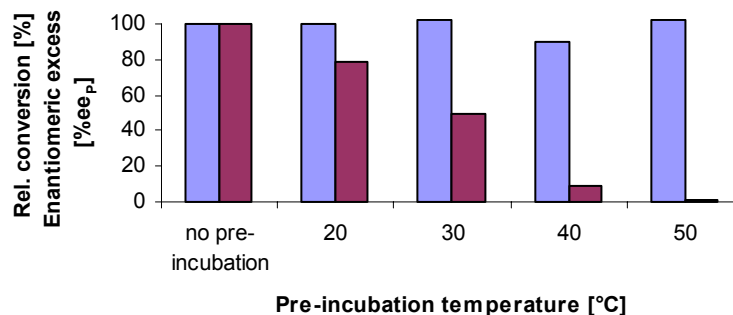


Figure 20. Determination of the temperature stability of Pf-ADH after 2 h pre-incubation of crude bacterial extracts. Acetophenone was used as substrate for the assays to determine residual activity. The blue bars represent the enantiomeric excess [%ee_p], the purple bars the relative conversion [%], the “no pre-incubation” sample values were set to 100% for calculations.

Addition of protease inhibitors did not result in stabilisation of enzyme activity (data not shown). This indicates, that the loss in activity is not due to protein degradation mediated by bacterial proteases. Some enzymes show reduced activity in diluted media because of a diffusive loss of stabilizing agents such as bovine serum albumin, glycerol, glucose or sucrose, which may help to protect enzyme structure, and enzymatic activity. In the case of formate dehydrogenase (FDH) the enzyme is chemically inactivated because of oxidation of cysteine residues with oxygen from air. Substitution of cysteine residues resulted in an increased operational stability, but at the same time a decrease of thermostability was observed (Tishkov and Popov 2004). For recombinant Pf-ADH, the reason for activity loss over time is not known. For optimal maintenance of activity the dissolved enzyme should be kept at 15°C. Stereoselectivity was not affected by preincubation of the enzyme at different temperatures.

4.9. Reduction of Acetophenone (Preparative Scale)

1.5 mmol Acetophenone (50 mmol/l final concentration) was dissolved in isopropanol (6.58 ml) and 0.1 M TRIS pH 8.0 (26.52 ml). The reaction was started by addition of lyophilized crude cell extract (420 mg). Due to the moderate stability of the Pf-ADH the activity decreased significantly within 24 h, so that further addition of crude Pf-ADH (final amount 3.2 g crude protein preparation) was necessary until 73% substrate conversion was achieved. The reac-

tion was carried out with stirring at room temperature for 355 h. After work-up of the reaction mixture (3x chloroform extraction with subsequent silica gel column chromatography, hexane:ethyl acetate 6:1, v/v), (*R*)- α -phenyl ethanol was isolated in 44% (660 μ mol) yield with enantiomeric excess of 92%*ee*_p. The fractions were analysed by TLC using hexane:ethyl acetate (6:1, v/v) as developing solvent.

4.10. Enzyme Purification

Enzyme purification from the lyophilised bacterial lysates was performed using metal affinity chromatography with TALON material. The Pf-ADH protein band at 32 kDa was detected in SDS-gels as expected, but also at least 10 weaker bands were detected in the Coomassie brilliant blue stained gel. The Pf-ADH presented the major protein band, but not as strong as expected from the crude protein extract data. The third washing step contained mainly Pf-ADH, but only in very low concentrations.

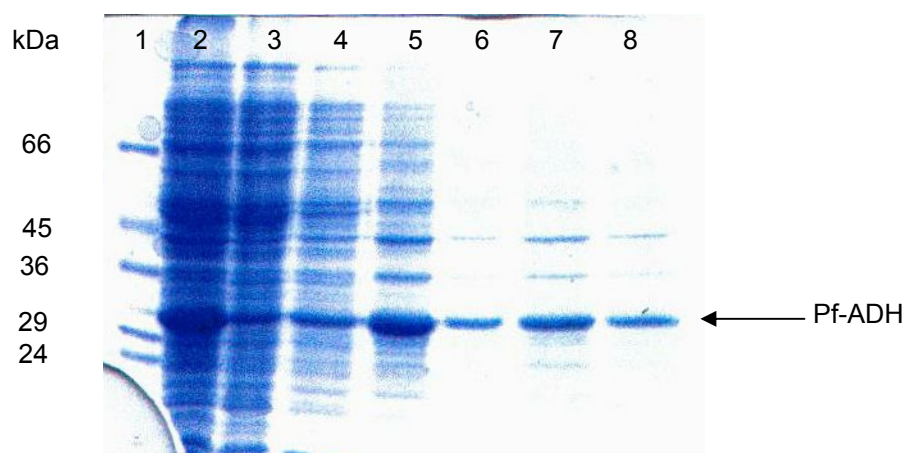


Figure 21. Coomassie-stained 12% SDS-gel, lane 1: low range molecular weight marker, lane 2: protein solution used for purification, lane 3: supernatant after binding to TALON cell thru material, lanes 4,5,6: washing steps with Na₂HPO₄-buffer pH 7.4, lanes 7,8: elution steps with Na-acetate buffer, pH 5.0.

From Figure 21 it becomes clear that a large amount of histidine-tagged protein is removed from the chromatography material already during the washing steps before elution. Hence, the final fraction contained only a small amount of the recombinant protein.

Since the yield of soluble protein was so low, it was attempted to prepare active enzyme by solubilising active enzyme from the pellet fraction of bacterial lysates. The pellet fraction after centrifuging the disrupted cells contained be-

sides mainly membrane-associated proteins also a significant amount of Pf-ADH stored in inclusion bodies. These inclusion bodies were solubilised by dissolving the protein extract pellet in 6 M guanidinium HCl containing denaturing buffer and applied this solution to the TALON material. Elution of bound protein was achieved using a low pH buffer (Figure 22).

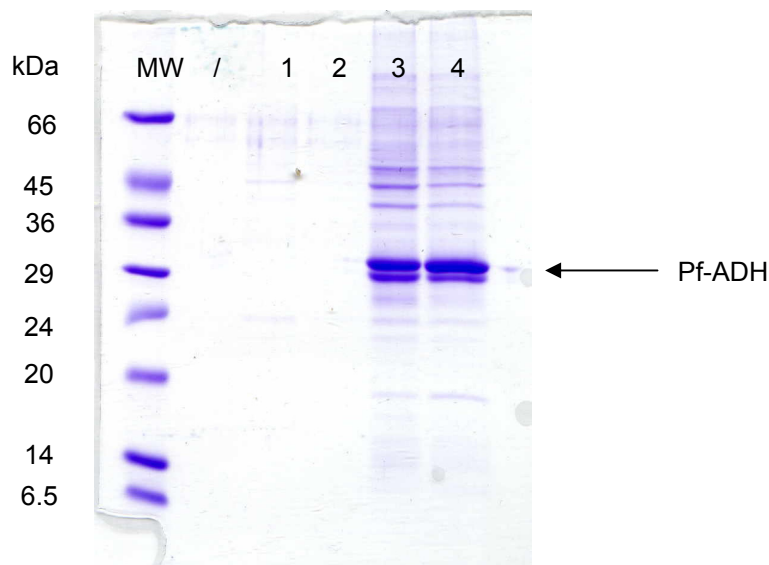


Figure 22. 12% SDS-gel displaying the result of the purification of Pf-ADH from guanidinium-HCl denatured pellet fraction, Lanes 1 and 2: elution with 8 M urea at pH 6.3, lanes 3 and 4: elution with 8 M urea pH 5.0.

Together with the recombinant Pf-ADH also numerous other proteins were co-eluted from the TALON material. The eluted protein fractions were tested in biocatalysis, but they showed no activity. To restore the catalytic activity of the Pf-ADH, refolding of the protein to recreate the secondary and tertiary structure would have been necessary. This step, however, was not attempted because in the elution from the TALON material no satisfactory purity of the enzyme was achieved.

In view of the fact that the Pf-ADH requires NADH as co-factor, experiments were performed to purify the enzyme from the soluble fraction of bacterial lysates using 5'-AMP-sepharose 4B. Since the 5'-AMP moiety has structural similarities to NADH binding of the enzyme to this material was likely. The first experiment which was done according to the manufacturers instructions (10 mM PB pH 7.4, 150 mM NaCl) did not result in the desired protein purification, because binding between the resin and the Pf-ADH did not occur (data not shown). To optimise the binding between Pf-ADH and Sepharose the optimal

pH and salt concentration were determined in small-scale samples. In these experiments, again no enrichment of Pf-ADH relative to other proteins by 5'AMP Sepharose was achieved. The majority of the Pf-ADH was found in the flow-through fractions, which indicated that the binding between the resin and the Pf-ADH did not occur. One reason for this could be the relative short contact time, when the sample is applied to the column. The other reason could be the presence of NADH in the protein preparation which inhibits the binding by competing with 5'AMP of the sepharose. Hence, the batch reaction was employed for the binding step (agitated for 1 h at room temperature on an orbital shaker) with a protein solution dialysed against PB, while the wash and elution steps were performed using a column. Kosjek et al. (2004) also tried the purification of the organic-solvent dehydrogenase from *R. ruber* with Blue Sepharose, a special stationary phase comparable to 5'AMP Sepharose, but the ADH did not bind and was eluted within the void volume.

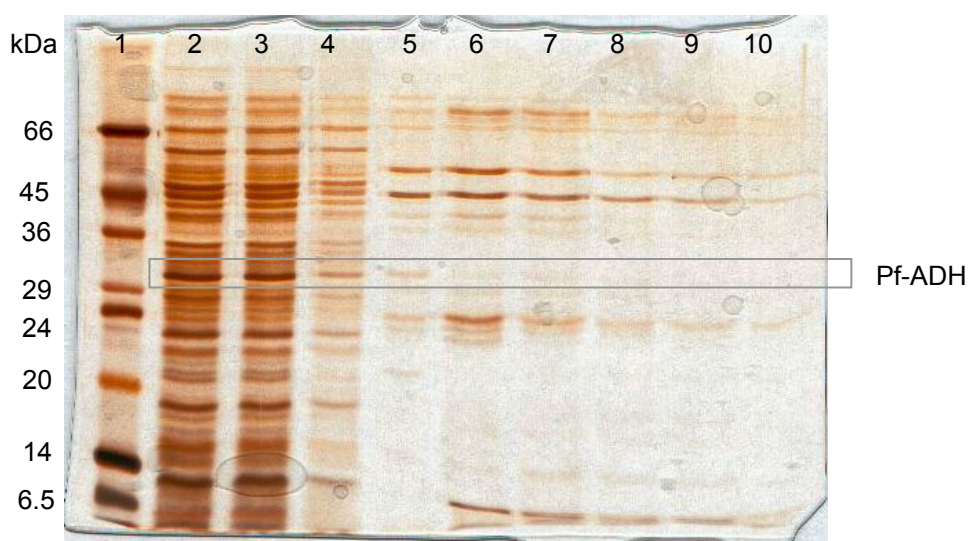


Figure 23. Silver-stained SDS-gel from the batch reaction with 5'AMP sepharose, lane 1: molecular weight protein standard, lanes 2 and 3: supernatant after binding, lane 4: wash step, lanes 5-10: eluted fractions.

As seen in Figure 23 the elongation of the reaction time and the use of NADH free protein solution did not lead to an efficient binding between Pf-ADH and 5'AMP sepharose. The binding site of NADH on the Pf-ADH may not be accessible for the 5'AMP of the chromatography material or the reaction site on the Pf-ADH, which usually accepts the dinucleotide from NADH, does not fit the mononucleotide present in the 5'AMP.

4.11. Substrate Specificity and Enantioselectivity

Besides acetophenone, which served as a model substance to optimize the reaction conditions and calculate the kinetic parameters, other compounds were investigated as possible substrates for reduction reactions. Here, aryl-substituted acetophenone derivatives as well as cyclic aliphatics, derivatives of cyclic aliphatics and 3-phenyl-butan-2-one, 3-oxo-butyric acid methyl ester, 3-oxo-hexanoic acid ethyl ester, and ethyl pyruvate were subjected to Pf-ADH-catalysed reactions.

Reaction mixtures consisted of 5 mg/ml crude protein, 50 mM substrate dissolved in isopropanol, 0.1 M TRIS pH 8.0, 20% isopropanol in 250 – 500 μ l reaction volume and were incubated at room temperature. GC analyses were performed at 200°C injection and 170°C detector temperature. The specific column temperatures for sufficient separation are listed in Tab.4.

Tab.4. Results for desymmetrisations of substrates 1-13 using recombinant Pf-ADH.

| Substrate/R ^a | E ^b [%ee _P] | Substrate conversion [%] | Notes | GC temperature program | Retention times ^c substrate/product(s) [min] |
|--------------------------|---------------------------------------|--------------------------------|--------------------------------|---|---|
| 1 | 92 | 95 | (R)-selective | 120° C isothermal or 120°C 2 min 10°C/min 150°C | 1.54/2.7/2.9 |
| 2 | 91 | 93 | Diastereomers not separated | 120°C 2 min 10°C/min 150°C | 3.9/5.05/5.69 |
| 3 | 92 | 91 | / | 120°C 2 min 10°C/min 150°C | 1.57/2.99/3.25 |
| 4 | 42 | 82 | / | 120°C 2 min 10°C/min 150°C | 2.61/3.49/3.77 |
| 5 | 44 | 37 | / | 120°C 2 min 10°C/min 150°C | 4.97/5.47/5.65 |
| 6 | 100 | 31 | Diastereomers not separated | 120°C 2 min 10°C/min 150°C | 3.99/5.51/5.69 |
| 7 | 78 | 28 | / | 120°C 2 min 10°C/min 150°C | 3.61/5.49/5.77 |
| 8 | 100 | >99 | / | 120°C 2 min 10°C/min 150°C | 1.59/2.16/2 nd poorly resolved |
| 9 | / | 51 | / | 90°C 1 min 5°C/min 150°C | 2.86/4.29 |
| 10 | 100 | 83 | Diastereomers not separated | 100°C isothermal | 0.69/0.82 |

| Substrate/R ^a | E ^b [%ee _P] | Substrate conversion [%] | Notes | GC temperature program | Retention times ^c substrate/product(s) [min] |
|--------------------------|---------------------------------------|--------------------------------|--------------------------------|---------------------------|---|
| 11 | / | 52 | / | 90°C 5°C/min 150°C | 0.95/1.37 |
| 12 | / | 100 | / | 90°C 5°C/min 150°C | 1.6/2.36 |
| 13 | 9 | 9 | Diastereomers not separated | 90°C 5°C/min 120°C | 0.91/1.43/1.54 |

^a R for 1-7 and 8-13 as depicted in Scheme 1; ^b enantiomeric excess, ^c the retention times are no fix values, because they depend on the actual configuration of the GC.

Initially, reductions using crude bacterial extracts and acetophenone as model substrate were performed in the presence of NADH. These reactions yielded (*R*)- α -phenyl ethanol. According to the mechanistic studies by Prelog, the dehydrogenase delivers the hydride ion from the *Si* face and thus shows anti-Prelog stereopreference (Prelog 1964). The expected anti-Prelog preference of the Pf-ADH was not confirmed by Poessl et al. (article in press 2005), for 1-chloro-2-octanone, ω -chloroacetophenone, and 3-chloro-1-phenyl-2-propanone. For these substrates the Pf-ADH acted according to the Prelog-rule and produced the (*S*)-enantiomers.

Conversions over 80% were obtained for acetophenone, ethyl butyrylacetate, 3-methoxy acetophenone, 4-fluoro acetophenone, 3-oxo-butyric acid methyl ester, and 2-methoxy acetophenone. Among these substrates only 2-methoxy acetophenone was converted with an enantiomeric excess [%ee_P] lower than 90, namely 30%ee_P. The excellent conversion of cyclohexanone in comparison with only 50% conversion of cyclopentanone and cycloheptanone and the very good conversion of acetophenone suggest that the size of the active site pocket can accommodate very well six-membered rings. The acetophenone derivatives substituted in *para*-position were converted less efficiently except for the 4-fluoro-acetophenone.

In addition to the substances listed above (Tab.4) also 3-phenyl-butan-2-one, 3-nitro acetophenone, 4-nitro acetophenone, 3-amino acetophenone, and 4-amino acetophenone were subjected to Pf-ADH-catalysed reduction reactions, but none of these were accepted as substrates.

To exclude the possibility that enzymes endogenously expressed by *E. coli* host bacteria affected some of these reactions, all substrates were subjected to

control reduction experiments, in which plain DH5 α protein extracts were used. Ethyl pyruvate was the only compound that was also accepted as substrate by the DH5 α strain lacking the plasmid and was completely metabolised by the host bacteria enzymes. Thus, from a practical point of view, the crude extract of the recombinant Pf-ADH resembles an extremely versatile catalyst as no addition of a second dehydrogenase, for example the frequently used formate dehydrogenase from *Candida boidinii* (Rissom et al. 1999), is required for recycling of NADH.

4.12. Kinetic Resolution of Racemic (*R,S*)- α -Phenyl ethanol

The dehydrogenase catalysed oxidative reaction can be employed for the kinetic resolution of racemic compounds. In highly stereospecific reactions, the enzyme leaves one isomer untouched and converts the other isomer completely into the ketone (yield of 50% of total material). The product can easily be separated from the reaction mixture by preparative chromatography.

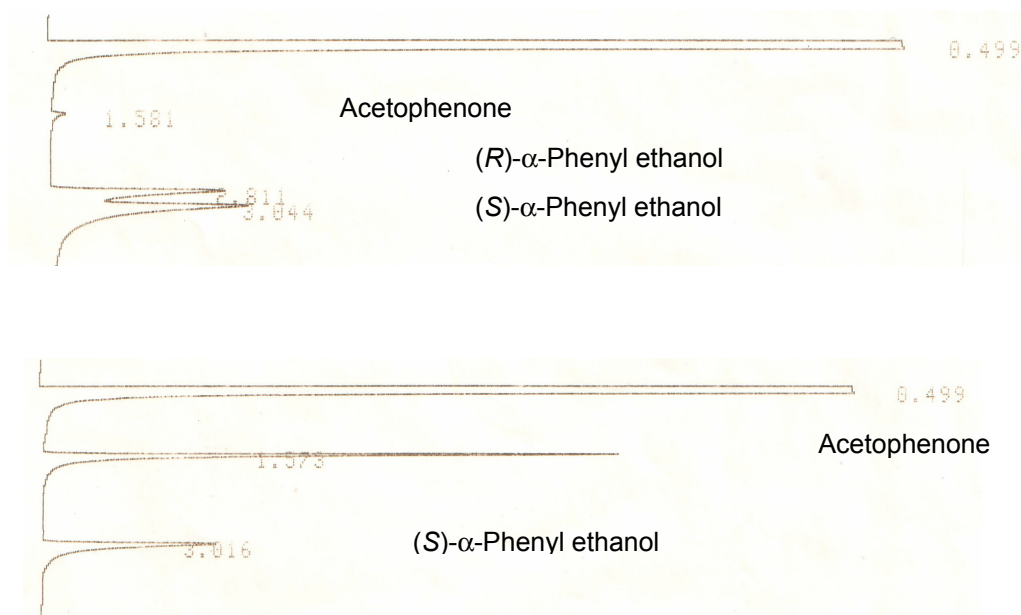


Figure 24. Kinetic resolution of (*R,S*)- α -phenyl ethanol. Top chromatogram: racemic (*R,S*)- α -phenyl ethanol ((*R*): rt 2.8 min, (*S*): rt 3.0min), bottom chromatogram: result of the kinetic resolution after 20 h: acetophenone (rt 1.6 min) and the (*S*)-enantiomer (rt 3.0 min) were detected.

Using the racemic mixture of (*R,S*)-phenyl ethanol as substrate for recombinant Pf-ADH, complete conversion of the (*R*)-enantiomer to acetophenone was observed (Figure 27), while the (*S*)-enantiomer was not accepted as a substrate at all and was, therefore, detectable in the reaction mixture after incubation

(Figure 27, lower panel) at approximately the same concentration as in the starting mixture (Figure 27, upper panel). The specific oxidative activity of Pf-ADH for α -phenyl ethanol was determined to $2 \text{ nmol min}^{-1} \text{ mg}^{-1}_{\text{prot}}$. This value is comparable to the data from the reduction mode, where the activity was found to be $1.1 \text{ nmol min}^{-1} \text{ mg}^{-1}_{\text{prot}}$.

4.13. Fluorimetric and Photometric Methods to Determine Dehydrogenase Activity

For routine analysis of potential substrates and stereoselective reactions, it would be useful to have one or more fluorimetric or photometric method, that reliably quantify enzyme activity towards specific substrates. Therefore, several methods were investigated for functionality and applicability in high-throughput assay systems.

Houdier et al. (1999) developed a method for the convenient and sensitive detection of aldehydes and ketones in aqueous samples. The carbonyls were derivatised using DNSOA (dansyloxyamine) giving a fluorescent complex, which can be detected. Since this method was compatible with aqueous samples, the derivatisation of acetophenone and α -phenyl ethanol was performed. The TLC analysis revealed that the reaction between DNSOA and acetophenone under the given conditions occurred as expected (data not shown). The eluted fractions from the silica gel chromatography were used to determine the optimal excitation and emission wavelength for fluorimetric measurements of DNSOA and DNSOA-acetophenone. The optimal excitation wavelength was determined to 420 nm for both compounds. Both substances showed an emission maximum at 542 nm, which made this method unsuitable to detect changes in DNSOA-acetophenone concentrations. The binding site of the acetophenone is separated from the fluorescent two-ring system by an oxime ether linker, which is probably too long to allow an influence of the bound moiety on the fluorescent part of the molecule.

The PMS/NBT reaction system (Figure 25) consists of four steps: a) oxidation of the substrate α -phenyl ethanol by Pf-ADH, b) thereby reduction of NAD^+ to NADH, c) reduction of the NBT *via* the PMS to form the water-insoluble formazan, and d) thereby regeneration of NAD^+ .

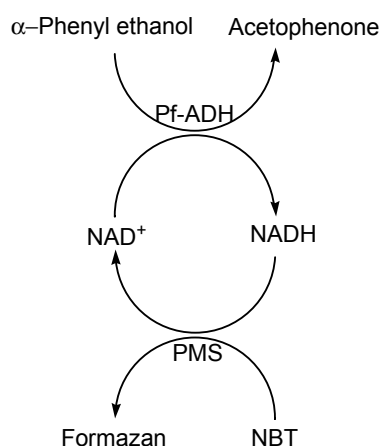


Figure 25. Reaction scheme of the PMS/NBT staining system used to detect oxidative alcohol dehydrogenase activity towards α -phenyl ethanol.

This system, which worked very well for the activity staining of the Pf-ADH in native gels, in renatured SDS-gels and on the isoelectric focussing strip, was used in microtiter plates with crude cell extracts. The non-specific background absorption measured by using lysates from the DH5 α strain without the Pf-ADH plasmid was equal or even higher after addition of the reagent PMS/NBT than for the Pf-ADH crude cell extracts. This reaction is probably due to other NADH-oxidising reactions. Therefore, this assay seemed not suitable to measure the activity of the recombinantly expressed Pf-ADH in crude protein preparations. The high background activity may be caused by the presence of isopropanol as co-solvent and as substrate for the NADH regenerating *E. coli* oxidoreductase. Omitting the isopropanol and adding the water-immiscible substrate directly to the aqueous reactions could solve this problem, but on the other hand, the conversion would be reduced, due to the limited substrate availability in aqueous solutions.

Another possibility seemed to be the potential spontaneous coupling of the hydrazine NBD-H to carbonyl groups. Control experiments, in which the NBD-H was dissolved solely in isopropanol, DMSO, isopropanol/water, or water already showed a steady increase of the relative fluorescence units (RFU). Using 1-propanol as organic solvent solved this problem, so that this technique was further explored.

A water-free 200 μ l assay volume contained equal amounts (100 nmol) NBD-H dissolved in 1-propanol and ketone or aldehyde (as control substances), respectively or alcohol. After incubation for 2 h at 50°C the RFU measured at

490nm_{ex}/520nm_{em} for acetaldehyde reached 20.000, for acetone 4.400 and for acetophenone 840. The control reaction, which contained merely NBD-H in 1-propanol, showed 400 RFU.

The resulting RFU for water containing samples were 13.000 RFU for acetaldehyde, 1.800 RFU for acetone, 900 RFU for acetophenone and 500 RFU for α -phenyl ethanol and the NBD-H control. The potential addition of an acidic catalyst (HCl or H₂SO₄) or increasing the temperature to increase reaction velocity was avoided, since this may result in the formation of unwanted fluorescent by-products (Gübitz et al. 1984).

Figure 26 gives an overview of the results, which were obtained with DMSO and DMF as co-solvents for better solubility of NBD-H and 1-propanol as general solvent. This shows that the presence of water (rows A, B, E, F in Figure 26) in the reaction mixture inhibited the formation of fluorescent reaction products with acetophenone and α -phenylethanol as substrates.

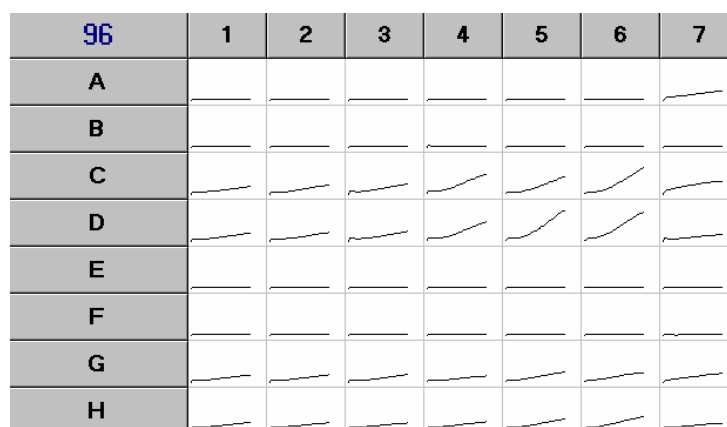


Figure 26. Overview of the fluorescence changes in different NBD-H conjugation assays: row A, B, E, F contained water (100 μ l), row A-D acetophenone, row E-H α -phenyl ethanol. Wells in row A, C, E, G contained NBD-H (100 nmol) dissolved in DMF, wells in row B, D, F, H NBD-H (100 nmol) dissolved in DMSO. Columns 1 to 6 contained the amount of 200, 400, 1200, 2400, 3600, and 4800 nmol of ketone or alcohol respectively. In all samples 1-propanol was used as organic solvent. Samples in column 7 served as negative controls and contained neither alcohol nor ketone.

In column 7, control samples were loaded containing neither alcohol nor ketone.

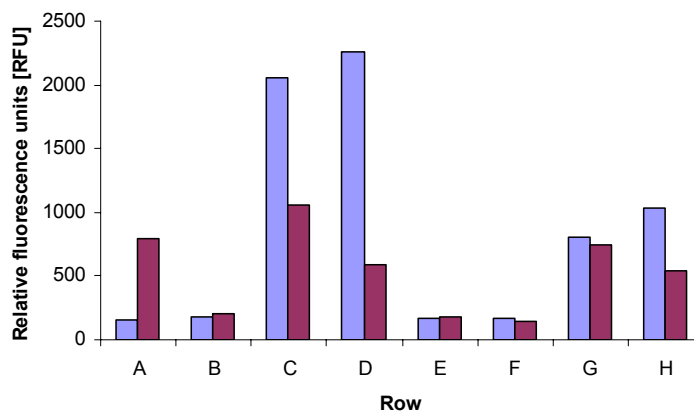


Figure 27. The maximum values from column 6 in Figure 26 (blue bars) are plotted next to the control samples from column 7 in Figure 26 (purple bars), which served as controls and contained neither alcohol nor ketone.

In Figure 27 the maximum reached RFU from the experiment shown in Figure 26 and the RFU from the corresponding negative control samples are presented. The RFU in the water containing samples in rows A, B, E, and F was not higher than the values of the respective negative controls.

For the screening of aqueous samples in high-throughput systems, this method is therefore not suitable, although it might be feasible for a reduced number of samples after an extraction step using organic solvents.

4.14. Polarimetric Measurement of Dehydrogenase Activity Towards Prostereogenic Substrates

Because the methods of fluorimetric and photometric techniques described above did not allow for high-throughput measurements of dehydrogenase activities, a polarimetric method was investigated as well, since the prostereogenic ADH-substrate acetophenone is optically inactive, while the respective product, α -phenyl ethanol occurs in optical active isomers, which are produced in different ratios by stereoselective enzymes. Such disproportional production of optically active substances is expected to change the rotation angle of polarized light.

The use of the Pf-ADH bacterial lysate in this assay was not possible, because the activity of this enzyme is too low and the necessary amount of lysate would cause a turbid solution. Measurement of turbid solutions will result in light scattering and this will lead to wrong readings (Gibbs et al. 2003).

For the polarimetric assay, the ADH from *Thermoanaerobacter spec.* (ADH TS) recombinantly produced in *E. coli* (3.34 U/mg_{lyo}, Juelich Fine Chemicals, Juelich, Germany) was used as a model enzyme. The ADH TS enzyme showed an intrinsic NADPH recycling activity, when isopropanol as hydrogen donor was present in the reaction mixture. The 2 ml reactions (50 mM TRIS pH 7.5, 0.1 mM NADPH, 10% isopropanol, 1 U ADH TS and 10 mM resp. 20 mM substrate) were set up in 24-well-plates and incubated for 1-2 h at room temperature prior to measuring.



Figure 28. POLARmonitor setup with 300 µl stainless cuvette (behind waste bottle), motor driven valve, peristaltic pump, liquid handler and keypad (from left to right).

After the incubation, the 24-well-plate was transferred to the working table of the liquidhandler, and the measuring program was started (Figure 28). The first sample, which contained no substrate, but buffer, isopropanol, NADPH and enzyme, was used to set the POLARmonitor reading to zero. Therefore, the first sample was pumped into the measuring cuvette and after the reading became stable, the autozero button was pushed. After ten seconds the next sample was automatically pumped into the cuvette, thereby removing the previous sample, which was discarded. This procedure was repeated until the optical rotation was determined for all samples.

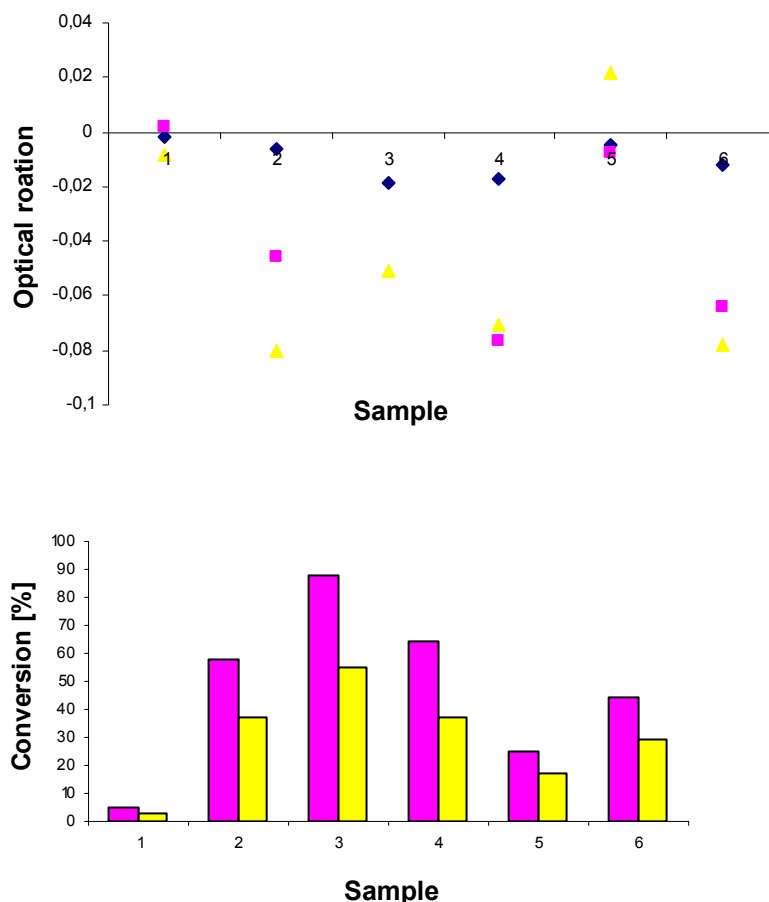


Figure 29. The top figure shows the optical rotation angles of products from ADH-reactions with different substrates. The bottom figure shows the corresponding conversion [%] as determined by GC. Substrates : 1: 3-methoxy-acetophenone, 2: 4-fluoro-acetophenone, 3: 4-chloro-acetophenone, 4: 4-methyl-acetophenone, 5: 2-methyl-cyclopentanone, 6: acetophenone. Pink symbols: 10 mM substrate, yellow symbols: 20 mM substrate. The blue symbols show polarimeter readings during cuvette washing with a reaction mixture without substrate between measurements, which demonstrate a constant background level.

As shown in Figure 29, the GC-analysis revealed a very low conversion for the 3-methoxy-acetophenone, which correlates very well with the optical rotation data. The optical rotation data for the 2-methyl-cyclopentanone (5) suggested no conversion, but the GC analysis revealed approximately 30% conversion. This indicates that the reaction was not enantioselective. For all other substrates tested, conversions between 30-90% were determined by GC analysis. These high conversions associated with the high enantioselectivity (> 99%*ee_P*) as indicated by the optical rotation data. This suggests that this method is suitable to measure the reaction velocity. Polarisation measurements

are obviously suitable to monitor the course of an ADH-reaction or determine end-points of reactions, given that the reaction is stereoselective and steady.

To test these prerequisites for a number of substrates, a set of three reactions were set up in a 24-well plate. Reactions were started by adding substrates and samples polarimetrically analysed at different times. To control conversion, GC analyses were performed on aliquots of the same assays.

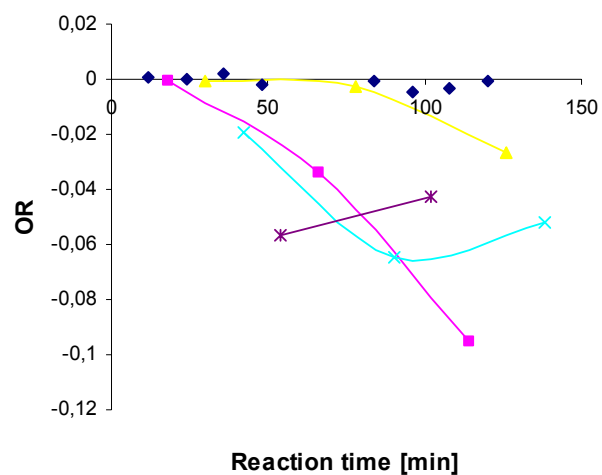


Figure 30.A

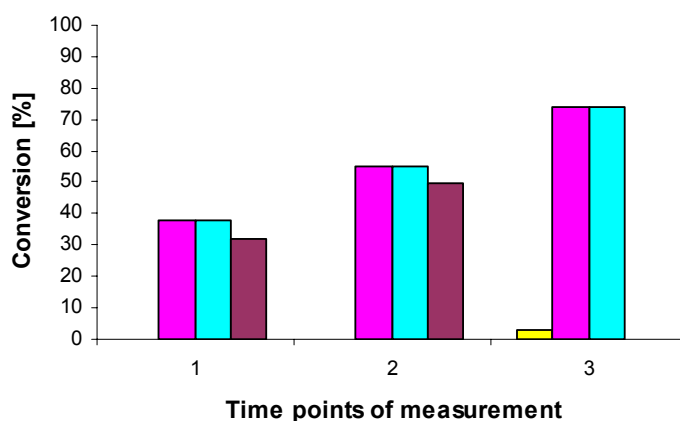


Figure 30.B

Figure 30 A and B Colour code: pink = acetophenone, light blue = 4-chloro-acetophenone, purple = 4-fluoro-acetophenone, yellow = 3-methoxy-acetophenone, dark blue = no substrate added; optical rotation data in Figure 28 A and corresponding GC-analysis in Figure 28 B, 10 mM starting concentrations of substrates.

The results in Figure 30 show that stereoselectivity and steadiness of the reaction are the two conditions, which were fulfilled for the substrates aceto-

phenone and 3-methoxy-acetophenone. 4-Chloro-acetophenone, however, showed a changed optical rotation direction as judged from the optical rotation at high total conversion.

A reason for this effect could be the delayed appearance of the second enantiomer. The racemic reaction product was not available to test, whether the single peak of the alcohol found by GC analysis contained one or both enantiomers.

As demonstrated by these examples, quantitative monitoring of a stereoselective reaction by polarimetry is theoretically possible, but it requires knowledge about the specific rotation values of the respective substrates under the given experimental conditions and requires 100% substrate conversion to calculate the enantiomeric excess. The two enantiomers of a product show the same specific optical rotation although in opposite directions. Therefore, the production of a racemic mixture would result in zero optical rotation despite 100% conversion. If the two enantiomers are produced at different rates, a net optical rotation will result that is the sum of individual rotation angles. Thus, the method will be most useful as a preliminary medium-through-put screening technique for identifying either a number of accepted substrates or different catalysts that show high stereoselectivity. Once the accepted substrates or stereoselective catalysts have been identified, their stereoselectivity can be measured more precisely, but also more time consuming, using chiral GC or HPLC.

5. Summary

In this work, the functional expression of a novel alcohol dehydrogenase (Pf-ADH) from *Pseudomonas fluorescens* DSM50106 is described, which was detected by sequencing a genomic library from this organism. The gene for the oxidoreductase was found in the neighbourhood of genes encoding for a cyclohexanone monooxygenase (BVMO) and an esterase. Assuming that these proteins are involved in the metabolic pathway, it was concluded that cyclic alcohols may be good substrates for the Pf-ADH, yielding the corresponding ketones, which are known to be converted by Baeyer-Villiger-monooxygenases (BVMO) to lactones. The esterase from this organism exhibits exceptionally high activity towards lactones (Khalameyzer et al. 1999).

The molecular mass calculated from the DNA of the Pf-ADH was identical to the molecular mass determined by SDS-page of the recombinant protein after detection of the Pf-ADH band with Ni-NTA AP conjugate.

The observation that renatured protein had enzyme activity after SDS-PAGE protein separation indicated that the enzyme may be also active as a monomer although it is likely that it occurs as a homodimer in *Ps. fluorescens*, as determined by native gel electrophoresis with subsequent activity staining.

The enzyme showed a temperature optimum at 15-20°C, a pH optimum at pH 8.0 and showed stable activity in the presence of up to 20% (v/v) isopropanol in the reaction mixture.

The k_m value of Pf-DH using acetophenone as a substrate was quite high indicating a relatively low affinity of the enzyme for this substrate. This may be part of the explanation for a relatively moderate conversion rate of V_{max} 6 nmol $min^{-1} mg^{-1}_{lyo}$ of this substrate. Substrate specificity, on the other hand, is not very high, since the enzyme efficiently reduces a broad range of ketones yielding the corresponding alcohols. An interesting feature in the activity of Pf-ADH is that it shows a high degree of enantioselectivity in the reduction of acetophenone, some of its derivatives, and 3-oxo butyric acid methylester, 3-oxo hexanoic acid ethylester and cyclic ketones and therefore can be considered as useful catalyst for the production of optically pure alcohols, which are used as building blocks in organic chemistry. The reduction of acetophenone by

Pf-ADH revealed an (*R*)-enantiomer preference of the enzyme, which thus shows anti-Prelog stereopreference. An inverted stereopreference of the Pf-ADH was found, when 1-chloro-2-octanone, ω -chloroacetophenone, or 3-chloro-1-phenyl-2-propanone were reduced (Poeschl et al. 2005).

Unfortunately, a simple way of protein purification was not found, but the crude protein extract from lysed host bacteria can be used in biocatalysis, since the host enzymes present in the preparation showed no activity towards the tested compounds with the exception ethyl pyruvate. The use of crude protein extracts has also the advantage that the addition of cofactor to the reaction is not necessary, because an endogenous *E. coli* alcohol dehydrogenase efficiently recycled NADH by oxidation of added isopropanol.

A polarimetric assay was developed to measure the activity of the ADHs towards prostereogenic ketones. This assay allows the medium-throughput-screening for oxidoreductases that exhibit good activity and high stereoselectivity. The method was developed using a partially purified ADH from *Thermoanaerobacter spec.* as a model enzyme, which is NADPH dependent and possesses an intrinsic cofactor regeneration capability, when isopropanol is present in the reaction mixture.

As mentioned above, Pf-ADH accepts cyclic ketones and might well be involved in the degradation of these substances in cooperation with the esterase and the Baeyer-Villiger-Monooxygenase (BVMO), whose genes were found in the vicinity of the alcohol dehydrogenase gene in the *Ps. fluorescens* DNA. To elucidate this context, further experiments with whole cell preparations may be useful.

Since the relatively low activity of the Pf-ADH may comprise use of this enzyme in industrial biotechnological applications, it may be useful to search for enzyme modifications that enhance conversion rates. This may be achieved by either site directed mutagenesis, which requires knowledge about the 3-dimensional protein structure, or by directed evolution using either bacterial mutator strains or employing error prone PCR.

6. List of Publications with Relevance to this Thesis

Hildebrandt, P., Riermeier, T., Altenbuchner, J., Bornscheuer U.T., Efficient resolution of prostereogenic arylaliphatic ketones using a recombinant alcohol dehydrogenase from *Pseudomonas fluorescens* DSM50106, 2001, Tetrahedron: Asymmetry **12**: 1207-1210

Hildebrandt, P., Musidlowska, A., Bornscheuer, U.T., Cloning, functional expression and biochemical characterization of a stereoselective alcohol dehydrogenase from *Pseudomonas fluorescens* DSM50106, 2002, Appl Microbiol Biotechnol **59**: 483-487

Patent applications: DE 101 12401 A1, EP 1 241 263 A1, Alkohol Dehydrogenase und deren Verwendung, T. Riermeier, U. Bornscheuer, J. Altenbuchner, P. Hildebrandt; US 2003/0171544 A1, Alcohol dehydrogenase and use thereof, T. Riermeier, U. Bornscheuer, J. Altenbuchner, P. Hildebrandt

Patent abstracts of Japan, 2002306189 A, Alkohol dehydrogenase Gene, vector, expression system, application of the alcohol dehydrogenase, and method for reductionally producing alcohol, T. Riermeier, U. Bornscheuer, J. Altenbuchner, P. Hildebrandt

Poessl, T.M., Kosjek, B., Ellmer, U., Gruber, C.C., Edegger, K., Faber, K., Hildebrandt, P., Bornscheuer, U.T., Kroutil, W., Non-racemic halohydrins via biocatalytic hydrogen transfer-reduction of halo-ketones and one-pot cascade-reaction to enantiopure epoxide, 2005, Adv Synth Catal (publication in press)

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8. Appendix

8.1. xTRAY Program to run the Liquidhandler and the peristaltic Pump

The xTRAY program file used to measure the 24-well-plates:

```
***** Programmlisting 24KINVS.XCP *****
** 08.07.2005 - 14:12:04
** Software : XTray
** Version : 4.031 / 12.01.2001
** GSIOC Ver: #GDE-engineer **

***** Systemkonfiguration *****

** ROBOTER : 221BV1.11 , ID: 10
   Z-Antrieb = 123 mm
   =====CONFIG/ROBOTER/ZDRIVE=====
   ZHome tiefer : 0      1/10mm
   LLD-Empfindl. : 2
   LLD-Offset   : 10     1/10mm

   =====CONFIG/ROBOTER/RINSE=====
   Spuelstation : custom
   max. Tiefe [mm]: 80      0..80
   custom-Abfluss : 516/ 262/1251 0.1mm
   custom-Spuelst.: 516/ 261/1251 0.1mm
   custom-Homepos.: 515/ 261/1251 0.1mm

   =====CONFIG/ROBOTER/VALVES=====
   ersetze LPV durch CON : 0 0/1..7

   =====Tray Konfiguration:=====
   Racks: Nr Pos Code xHome yHome zHome W. M.
         1 r æT024 594 544 278 1

   Zonen: Zonenname  Vials  ? Sum  Rack
   REAGENT_A    1-1  -  1 1-1
   SAMPLE_B     2-24  ?  23 1-1

** MINIPULS : 312 , ID: 30

***** Programm *****

1 MoveToZone HOME
2 SetContact Nr: 6 PULS
3 MoveToZone CusRinse
4 PumpMP3 0 s CCW ->CusRinse
5 BeginLoop
6 ^RecordData SAMPLE_B 0809041.DAT
7 ^MoveToZone SAMPLE_B
8 ^PumpMP3 250 s CCW ->SAMPLE_B Pump sample into cuvette
9 ^RecordData SAMPLE_B 0809041.DAT
10 ^Wait 60 s Allow sample to calm down
11 ^RecordData SAMPLE_B 0809041.DAT record data 6 times every second
12 ^Wait 1 s
13 ^RecordData SAMPLE_B 0809041.DAT
```

```
14 ^Wait      1 s
15 ^RecordData SAMPLE_B 0809041.DAT
16 ^Wait      1 s
17 ^RecordData SAMPLE_B 0809041.DAT
18 ^Wait      1 s
19 ^RecordData SAMPLE_B 0809041.DAT
20 ^Wait      1 s
21 ^RecordData SAMPLE_B 0809041.DAT
22 ^Wait      1 s   After this step the cycle starts again
```

8.2. Nucleotide and Amino Acid Sequence of the *adhf1* Gene and the Pf-ADH Protein

Nucleotide sequence of the alcohol dehydrogenase coding gene *ADHf1* from *Pseudomonas fluorescens* DSM50106

Accession number: BD177958

```
1  atgaagtcac tcaacggccg cgtggcggcg attaccggcg cggcatccgg catgggtcgc
61  gcattggccc tggcactcgc gcgcgaaggt tgccacctgg cactggcgga caaaaacgcc
121 caaggcctgg agcagaccct ggcactgatc aagacctcga ccctgtcgcc ggtgatggtc
181 accaccaggg tgctggatgt ggccgaccgc caggccatgg aggcttgggc ggcgcgctgc
241 gtggccgagc atggccaggt caacctgggt ttcaacaacg ccggcgtggc cctgtcgagt
301 acggtcgaag gctgggacta cgccgacctg gactggatcg tcggcatcaa cttctggggc
361 gtggtccacg gcaccaaggc gttcctgccg cacctcaagg ccagcggcga cggccatgtg
421 atcaacacgt ccagcgtggt cggcctgttt gccagccccg gcatgagcgg ttacaacgcg
481 accaaattcg ccgtgcgcgg ctttaccgaa gccctgcgcc aggagctgga cctgcaacgc
541 tgcggcgtct cggccacctg cgtgcacccc ggcggcatcc gcaccgatat ctgtcgcagc
601 agccgcatcg acgcgaacat gaccggcttc ctgatccaca gcgaacagca ggcccgcgcc
661 gacttcgaaa aactcttcat caccgatgcc gaccaggccg ccaaggtgat cctgcaaggc
721 gtccgcaaaa acaagcgtcg cgtgctgata ggccgcgacg cgtatttctt cgacctgctc
781 gcccgttgcc tgccggcggc ctatcaagcg ctggtggtgc tggccagcaa gcgcatggcc
841 cccaagcaac gcaggccagt gtttgaacc aacgacgagc cccgtctctg a
```

Amino acid sequence of the product of the *adhf1* gene encoding the Pf-ADH protein

```
/protein_id="AAL79772.1"
/db_xref="GI:18860822"
/translation=
```

```
MKSFNGRVAAITGAASGMGRALALALAREGCHLALADKNAQGLE
QTLALIKTSTLSPVMVTTQVLDVADRQAMEAWAARCVAEHGVNLFVFNAGVALSSTV
EGVDYADLEWIVGINFWGVVHGTKAFLPHLKASGDGHVINTSSVFGLFAQPGMSGYNA
TKFAVRGFTEALRQELDLQRCGVSATCVHPGGIRTDICRSSRIDANMTGFLIHSEQQA
RADFEKLFITDADQAAKVILQGVKRNKRRVLIGRDAYFLDLLARCLPAAYQALVVLAS
KRMAPKQRRPVFETNDEPRL
```

8.3. Curriculum Vitae

| | |
|---------------------|---|
| Name: | Petra Hildebrandt, née Plischke |
| Birthday: | May 14, 1958 |
| Place of birth: | Hannover, Germany |
| Parents: | Margot Plischke, née Volkmann and Reinhard Plischke |
| 1965-1968: | Elementary school Misburg, Niedersachsen |
| 1968-1975: | Gymnasium Schillerschule Hannover |
| 1975-1978: | Integrierte Gesamtschule Roderbruch, Hannover |
| June 13, 1978: | Final Examination (Abitur) |
| October 1981: | Study of Biology at Freie Universität Berlin |
| September 27, 1985: | Marriage with Jan-Peter Hildebrandt |
| April 1986: | Birth of twin boys, Florian and David |
| July 1988: | Final examination (Diplomprüfung Biologie) |
| 11/1988-1/1990: | Werkvertrag at the Robert-Koch-Institut, Bundesgesundheitsamt (Monoclonal antibodies against non-structural HIV proteins) |
| 1/1990- 1/1993: | University of Rochester, NY, USA; research assistant in Prof. D.J. McCance's lab |
| 2/1993: | Move back to Germany, Zweibrücken |
| 9/1993 – 7/1996: | Wissenschaftliche Mitarbeiterin at the Institute for Anatomy, Universität Homburg, Saarland, Prof. P. Mestres |
| 8/1996 – 2/2000: | free lance activity as information broker for the medical and scientific community |
| 3/2000 – 5/ 2002: | Wissenschaftliche Mitarbeiterin at the Institute for Chemistry & Biochemistry, Technical Chemistry & Bio- technology, University of Greifswald, Mecklenburg- Vorpommern, Prof. U. T. Bornscheuer |

-
- 6/2002 – 4/2003: Attendance of the full-time practical course “Methods in Molecular Biology” offered by the Forschungsverbund Mecklenburg-Vorpommern (FMV)
- 5/2003 – 2/2005: Wissenschaftliche Mitarbeiterin at the Institute for Chemistry & Biochemistry, University of Greifswald, Prof. U. T. Bornscheuer