

Cloning, expression, characterization and immobilization of carbonyl reductase from *Candida parapsilosis*

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Table of Contents

Table of Contents	I
Glossary of Abbreviations and Symbols	IV
1 Introduction	1
1.1 Chirality and biocatalysis	1
1.2 Alcohol dehydrogenases	2
1.3 Carbonyl reductase from <i>Candida parapsilosis</i>	2
1.4 Versatility of CPCR to other alcohol dehydrogenases	4
1.5 Secondary alcohol dehydrogenase from <i>Candida parapsilosis</i>	5
1.6 Cofactor regeneration	6
1.7 Alcohol dehydrogenases in organic solvents	8
1.8 Immobilization	10
1.9 Aim and motivation of the research work	15
2 Materials and Methods	17
2.1 Materials	17
2.1.1 Chemicals	17
2.1.2 Devices	18
2.1.3 Cultivation media	19
2.1.4 Microorganisms	20
2.1.5 Vectors	21
2.1.6 Materials for Molecular biology work	21
2.2 Microbiological methods	22
2.2.1 Conservation and storage of microbiological strains	22
2.2.2 Cell cultivation	23
2.2.3 Cell disruption and preparation of cleared <i>E.coli</i> lysates under native conditions	24
2.2.4 Preparation of competent cells of <i>E.coli</i>	25
2.3 Molecular biology methods	27
2.3.1 Preparation of <i>C.parapsilosis</i> genomic DNA (gDNA)	27
2.3.2 Construction of genomic DNA libraries	27
2.3.3 Synthetic DNA-oligonucleotides	28
2.3.4 Polymerase chain reaction (PCR)	29
2.3.5 Agarose gel electrophoresis	32

2.3.6	Plasmid DNA isolation from <i>E.coli</i>	33
2.3.7	DNA restriction digestion	34
2.3.8	5' Dephosphorylation of DNA fragments	34
2.3.9	Ligation of DNA fragments	34
2.3.10	Transformation and selection	35
2.3.11	Preparation of DNase free RNase	36
2.3.12	Sequencing	36
2.3.13	Description of cloning set-up of recombinant CPCR and CpSADH	37
2.4	Biochemical and analytical methods	37
2.4.1	Determination of protein	37
2.4.2	Determination of enzyme activity	38
2.4.3	Protein purification	40
2.4.4	Gel electrophoresis	43
2.4.5	Transfer of protein (Western Blot)	44
2.4.6	Chromatographic analysis of substrate and product	45
2.4.7	Inhibition study	45
2.4.8	Temperature optimum and activation energy	46
2.4.9	Temperature stability and half-life	46
2.4.10	pH optimum and pH stability	47
2.4.11	Stability in hexane	47
2.4.12	Synthesis in aqueous system	48
2.4.13	Synthesis in liquid two-phase system	48
2.4.14	Substrate spectrum of recombinant CpSADH	49
2.5	Immobilization of recombinant CpSADH	49
2.5.1	Immobilization in polyvinyl alcohol (PVA)	49
2.5.2	Immobilization in Sepabeads	50
2.5.3	Stability of the immobilized CpSADH in Sepabeads	52
3	Results and Discussion	53
3.1	Cloning of CPCR and CpSADH	53
3.1.1	Isolation of genomic DNA from <i>C.parapsilosis</i>	53
3.1.2	Construction of libraries	53
3.1.3	Analysis of the partial amino acids sequences of wtCPCR	54
3.1.4	PCR amplification of CPCR from the libraries and subcloning in pDrive vector	57

3.1.5	Cloning of the 1212 bp CPCR gene into expression vectors	66
3.1.6	Cloning of the 1011 bp CpSADH gene into expression vectors.....	69
3.1.7	Conclusion of the cloning of CPCR and CpSADH.....	75
3.2	Expression of recombinant CPCR and CpSADH	77
3.2.1	Expression of recombinant CPCR.....	78
3.2.2	Expression of recombinant CpSADH	80
3.2.3	Conclusion of the expression of recombinant CPCR and CpSADH	90
3.3	Characterization of recombinant CpSADH.....	91
3.3.1	Temperature optimum and temperature stability	91
3.3.2	pH optimum and pH stability	94
3.3.3	Stability in hexane	95
3.3.4	Inhibition study	97
3.3.5	Substrate spectrum of recombinant CpSADH	98
3.3.6	Conclusion of the characterization of recombinant CpSADH.....	102
3.4	Computer modeling and simulation of CPCR and CpSADH	103
3.4.1	Conclusion of the modeling data.....	106
3.5	Biocatalytic synthesis in different systems	106
3.5.1	Enzymatic synthesis in an aqueous system.....	107
3.5.2	Enzymatic synthesis in a liquid two-phase system	108
3.5.3	Conclusion of the enzymatic synthesis in different liquid systems	112
3.6	Immobilization of recombinant His-tagged CpSADH and enzymatic synthesis with the immobilisate	112
3.6.1	Immobilization in PVA and enzymatic synthesis thereof.....	113
3.6.2	Immobilization in Sepabeads and enzymatic synthesis thereof.....	115
3.6.3	Conclusion of the immobilization	128
4	Summary.....	129
5	References	131

Glossary of Abbreviations and Symbols

ABEE	Acetyl-butyric-acid-ethyl ester
ADH(s)	Alcohol dehydrogenase(s)
amp	Ampicillin
bp	Base pairs
Conc.	Concentration
CPCR	<i>Candida parapsilosis</i> carbonyl reductase
CpSADH	<i>Candida parapsilosis</i> secondary alcohol dehydrogenase
C-terminal	Carboxy-terminal
Da	Daltons
DAB	3,3-Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FDH	Formate dehydrogenase from <i>Candida boidinii</i>
FPLC	Fast Protein Liquid Chromatography
x g	Relative centrifugal force (gravitational force)
GC	Gas Chromatography
gDNA	Genomic DNA
HHEE	Hydroxy-hexane-acid-ethyl-ester
HLADH	Horse liver alcohol dehydrogenase
HPLC	High Pressure Liquid Chromatography
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl- β -D-thiogalactopyranoside
kan	Kanamycin
kb	Kilo base (pairs)
kDa	Kilo Daltons
LB	Luria Bertani medium
LKADH	<i>Lactobacillus kefir</i> alcohol dehydrogenase

MW(CO)	Molecular weight (cut-off)
NAD ⁺	β-nicotinamide adenine dinucleotide, oxidized form
NADH+H ⁺	β-nicotinamide adenine dinucleotide, reduced form
N-terminal	Amino-terminal
NTA	Nitrilotriacetic acid
OD	Optical Density
ORF	Open reading frame
PADH/PED	<i>Pseudomonas sp.</i> alcohol dehydrogenase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonylfluoride
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene fluoride
RNAse	Ribonuclease
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA
TB	Terrific broth medium
TBADH	<i>Thermoanaerobium brockii</i> alcohol dehydrogenase
TBS	Tris-buffered-saline
TE	Tris-EDTA
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
T _m	Melting temperature
TTN	Total turnover number, it is a dimensionless quantity
U	Units
UV	Ultra violet
v/v	Volume per volume
wtCPCR	Wild type <i>Candida parapsilosis</i> carbonyl reductase
YADH	Yeast alcohol dehydrogenase
ε	Extinction coefficient of cofactor (l mmol ⁻¹ cm ⁻¹)
τ	Half-life (h)

1 Introduction

1.1 Chirality and biocatalysis

The biological activity of a chiral compound depends strongly on the stereochemistry of the molecule. While one enantiomer has a desired therapeutic effect, the other can have no or even an opposite effect. The most well known example for this is the medicine Contergan® containing the active substance thalidomide, the (*S*)-enantiomer of which possesses a teratogenic effect. Therefore, a high enantiomeric purity is necessary particularly in the pharmaceutical, agrochemical and food industries. There is an increasing trend in these industries, to develop products containing enantiomerically pure materials. This trend was accelerated by the decision of the American Food and Drug Administration (FDA) in May 1992. Safety information is now demanded for individual stereoisomers of products submitted for approval and although racemates will still be continued to be approved on a case-by-case basis, information on each of the enantiomers is required (Peters, 1998). Chiral compounds can be produced by either chemical synthesis (which is not necessarily catalytic) or biocatalysis (using either whole cells or isolated enzymes). Biocatalysis has been a key focus area in white biotechnology (application of nature's toolset to industrial production) (Bachmann, 2003). A recent report of McKinsey predicted that by the year 2010, white biotechnology would be a competitive way of producing about a fifth of world's fine chemical segments (Bachmann, 2003). According to another recent study from Frost and Sullivan it is expected that biocatalysis will increase its share from 10% in 2002 to 22% in 2009 of the annual turnover for chiral technologies (Widmer, 2003). This is because of the growing use of enzymes as substitutes for conventional chemical catalysts in production processes, for example in the detergent industry, food and pharmaceutical industries (Liese and Filho, 1999). Biocatalysts offer some advantages over chemical catalysts and these include necessity of only mild reaction conditions, avoidance of unwanted by-products (when isolated enzymes are used) (Liese and Filho, 1999), and of course a high chemoselectivity, regioselectivity and especially stereoselectivity for the production of enantiomerically pure compounds that may not be necessarily exhibited by the chemical catalysts. Biocatalytic resolutions of racemates make use of the selectivity of the enzymes for one of the enantiomers of the chiral molecule, whereby one enantiomer of a racemate remains virtually untouched and the other enantiomer is converted into the desired enantiomerically pure product/intermediate. As a result of the growth in demand for chiral actives, the market for

chiral intermediates is growing fast. This has provided an enormous impetus for the development of enantioselective chemical and biochemical transformations, such as the reduction of carbonyl compounds and/or oxidation of alcohols. Biocatalytic applications of oxidoreductases for such transformations have been an important tool in biotechnology. In particular, alcohol dehydrogenases (ADH) are increasingly being used for asymmetric synthesis, due to the growing demand for enantiomerically pure pharmaceutical compounds (Bradshaw, et al. 1992a; Wong and Whitesides, 1994; Hummel, 1999).

1.2 Alcohol dehydrogenases

Alcohol dehydrogenases (EC 1.1.1.1) are enzymes that belong to the first sub-class of the oxidoreductase family, which catalyze the oxidation of primary and secondary alcohols and/or reduction of carbonyl compounds like aldehydes and ketones. An important characteristic of alcohol dehydrogenases (ADH) is their dependence on $\text{NADH}+\text{H}^+$ and/or $\text{NADPH}+\text{H}^+$ as cofactors.

An important thing to consider when using alcohol dehydrogenase is whether they follow the Prelog or the anti-Prelog rule (Prelog, 1964), which states from which side the hydride transfer takes place (*si*- or *re*- side) so as to determine whether the enantiomer formed is (*R*) or (*S*) specific. Many alcohol dehydrogenases are available in nature but they vary specially in their cofactor dependence, stereospecificity and substrate spectrum. The stereospecific reduction of carbonyl compounds is of great interest for the production of intermediates of chiral compounds like hydroxy acids, amino acids and alcohol and prochiral intermediates. Such products can find direct applications in food or as components in the synthesis of therapeutics, herbicides and pesticides.

1.3 Carbonyl reductase from *Candida parapsilosis*

In the year 1992, Peters et al. reported for the first time an oxidoreductase from the yeast *Candida parapsilosis* DSMZ 70125 strain. This novel, strictly NAD(H)-dependent, carbonyl reductase (CPCR), has an extremely broad substrate range and high enantioselectivity, accepting primary and secondary alcohols, aldehydes, ketoacetals, aliphatic and aromatic ketones, cyclic ketones, diketones, halogenated ketones, keto esters and halogenated keto esters of variable chain length as substrates (Peters et al., 1992, 1993a; Kula et al., 1996). The enzyme oxidizes primary and secondary alcohols and is able to differentiate between the

enantiomers of 2-butanol, which is difficult with other alcohol dehydrogenases (Keinan et al., 1986b, 1987). CPCR delivers the pro-(*R*) hydride of the nicotinamide ring to the *re* face of the carbonyl yielding (*S*)-hydroxy compounds (Peters et al., 1993b). Thus the stereochemical outcome follows Prelog's rule. The substrate spectrums that had been identified for the CPCR were all converted with (*S*)-configuration and with a very high enantiomeric purity (Peters et al., 1993a,b, c; Rissom, 1999). CPCR was successfully applied for the preparation of a range of synthetically useful chiral compounds (Peters et al., 1993c, d).

Literature data available regarding the characteristics of this novel enzyme varies widely. Initially this enzyme was characterized as a homodimer enzyme with a molecular weight of about 135-136 kDa (Kula et al., 1996; Peters et al., 1993a). Later, in newer publications, the molecular weight of the native CPCR was described to be approximately 78-79 kDa with subunit size of about 40 kDa per subunit (Rissom et al., 1999; Orlich, 2000; Bönitz, 2001; Laue, 2001). The enzyme is described to be found in the peroxisomes of the yeast cells. The biological function of the described enzyme in *C.parapsilosis* seems to be the reduction of carbonyl compounds due to its kinetic characteristics (Peters et al., 1993a). However, the natural substrate of the enzyme is still unknown. The enzyme may be involved in fatty acid metabolism and/or regeneration of NAD^+ from $\text{NADH}+\text{H}^+$ (Peters, 1993; Peters et al., 1993a). The enzyme is characterized by temperature optima between 36 °C to 40 °C (reduction) and 50 °C to 56 °C (oxidation), while the pH optima are 7-8.5 and 9-10.5 for reduction and oxidation reactions respectively (Peters et al., 1993a, Peters 1993; Kula and Peters, 1996; Bönitz, 2001). Very recent characterization of the CPCR showed that the temperature optimum for reduction was 48 °C and the pH optimum was 6 instead (Steinsiek, 2006).

Based on the substrates that had been investigated for CPCR, a hypothetic model of the substrate-binding site has been deduced from the kinetic measurements (Peters et al., 1993b; Kula et al., 1996). CPCR possibly possesses two hydrophobic substrate-binding sites, which differ from one another in volume and affinity towards the alkyl groups. One side chain of the keto group has to be a small alkyl group while the other group may be a large hydrophobic side chain. From this model, the substrate spectrum of CPCR was concluded. Very polar substances, which do not possess a similar methyl side chain, could not be reduced (Figure 1).

The catalytic cycle follows a sequentially ordered bi-bi-mechanism (Cleland, 1986). First the cofactor binds to the CPCR enzyme, followed by the binding of the substrate. After the substrate is converted, the product is released from the binding site and afterwards, the oxidized cofactor is set free.

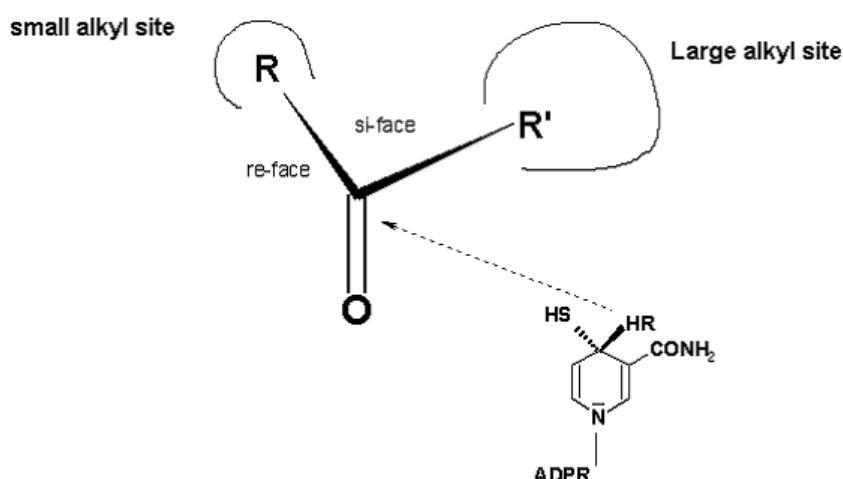


Figure 1: Hypothetical structure of the CPCR substrate-binding site. ADPR = Adenosine 5' diphosphate ribose.

1.4 Versatility of CPCR to other alcohol dehydrogenases

The CPCR has a wide range of advantages compared to other alcohol dehydrogenases. Hummel and Kula (Hummel et al., 1989) discussed different alcohol dehydrogenases and their applications. Alcohol dehydrogenases from yeast (YADH) and horse liver (HLADH) have several disadvantages, such as instability above 30 °C, sensitivity to organic solvents, loss of activity upon immobilization and narrow substrate specificities (Keinan et al., 1986a, b; Sih and Chen, 1984). Disadvantages of some alcohol dehydrogenases that were isolated from certain thermophilic microorganisms like *Sulfolobus solfataricus* and *Thermoanaerobium Brockii* are moderate specific activity (3.9 U/mg) and NADPH dependency respectively. A NADH-dependent alcohol dehydrogenase from *Pseudomonas sp.* (PED) showed broad substrate specificity (Bradshaw et al., 1992a). But one disadvantage of this enzyme is the high K_m value for the oxidized coenzyme. Both PED and the NADPH-dependent *Lactobacillus kefir* alcohol dehydrogenase (LKADH) yield the (*R*)-enantiomer

(Hummel, 1990a, b) while the CPCR produces the (*S*)-enantiomer and is NADH-dependent. The ketopantoyl reductase (Hata et al., 1989) and carbonyl reductase (Hata et al., 1990) isolated from another strain of *C.parapsilosis* are NADPH-dependent enzymes inactive with keto acids or esters as substrates. 4-Ketoacids or esters were not reduced by PED. LKADH reduces 4-keto esters at much lower rates (Bradshaw et al., 1992b). In contrast, the NADH-dependent CPCR reduces 4- and 5-keto esters of variable chain length and substitution at high reaction velocities and enantioselectivity (Peters et al., 1993a,b). The alcohol dehydrogenases from *Thermoanaerobium brockii* (TBADH) and *Pseudomonas sp.* (PED and PADH) generally require that one of the ketone side chains be a methyl group (Bradshaw et al., 1992a); whereas CPCR accepts a broad spectrum of side chains, including aromatic and cyclic groups as well as halogen-substituted carbon chains. Although the substrate specificity of CPCR is partially overlapped with PADH, PED and TBADH, most substrates are reduced at higher rates by the CPCR, especially the reduction of synthetic useful acetophenone derivatives and 4-chloro-3-oxobutanoate (Peters et al., 1993a). The CPCR showed the possibility of converting acetophenone and its derivatives to the corresponding (*S*)-phenylethanol in NADH-dependent catalysis, which is especially interesting since up to now only the (*R*) specific transformations could be realized.

1.5 Secondary alcohol dehydrogenase from *Candida parapsilosis*

In the year 1995, Kojima et al. reported for the first time a novel secondary alcohol dehydrogenase from the yeast *Candida parapsilosis* IFO 1396 strain. The gene coding for this secondary alcohol dehydrogenase (CpSADH) was isolated, cloned and expressed in the *Escherichia coli* JM109 by the same group. CpSADH catalyzes the NADH-dependent reduction of ketones to the corresponding secondary alcohols. The enzyme catalyzes the enantioselective oxidation of racemic secondary alcohols and the asymmetric reduction of aromatic and aliphatic ketones to their corresponding secondary alcohols with (*S*) selectivity (Yamamoto et al., 1995). Furthermore, the said enzyme preferentially oxidizes (*S*)-2-butanol with the *S* configuration. The said enzyme has a relative higher activity toward secondary alcohols than primary ones. This novel enzyme has very broad substrate specificity, good stereoselectivity and utilizes aliphatic alcohols including those with an aromatic substitution as its oxidizing substrate as well as aldehydes and ketones with an aromatic substitution. The recombinant CpSADH has been extensively used for whole cell biocatalysis for the synthesis of several pharmaceutical intermediates for industrial use with a very high enantiomeric purity (Matsuyama et al., 1993; Yamamoto et al., 1999; Matsuyama et al., 2001; Matsuyama

et al., 2002; Yamamoto et al., 2002). The enzyme is characterized with a temperature optimum of 50 °C and pH optima of 5.5-6.5 (reduction) and 8.5-9.5 (oxidation). The values of the temperature optimum and the pH optimum for reduction of CpSADH matches exactly to what has been described by Steinsiek (2006) for the wild-type CPCR. The apparent molecular weight of the enzyme is estimated to be about 40 kDa by SDS-PAGE.

1.6 Cofactor regeneration

Due to the high costs of the nicotinamide cofactors, in order to avoid a stoichiometric amount of the cofactor, cofactor regeneration is necessary for the economic production of chiral compounds with the help of alcohol dehydrogenases so that only catalytic quantities of the reduction equivalents can be used. Cofactor regeneration can be carried out with enzymatic (biological) or non-enzymatic methods. With the biological method, whole cells or isolated enzymes can be used for the regeneration of the cofactors. The non-enzymatic method is differentiated into chemical, electrochemical and photochemical methods (Chenault et al., 1988). The chemical method uses a reducing agent like sodium dithionite and therefore suffers from a very low total turnover number of less than 100 (Jones et al., 1972) and the reducing agent can also deactivate enzymes (Ramio and Lilius, 1971). The electrochemical method uses electricity and the photochemical method uses light energy for the cofactor regeneration. The major disadvantages of most electrochemical and photochemical regeneration methods are low regioselectivity (Mandler and Willner, 1986; Jones and Taylor, 1976; Legoy et al., 1980; Julliard et al., 1986), leading to coenzyme/cofactor inactivation, occurrence of side reactions, and low total turnover numbers of less than 1000 (Chenault and Whitesides, 1987; Chenault et al., 1988). Another disadvantage of the electrochemical regeneration method is the fouling of the electrodes by adsorption of the substances from the solution. For industrial applications, cofactor regeneration system must fulfill certain requirements and these include apart from a high reaction rate and a high conversion, stability of the enzyme and the cofactor in the reaction system. The efficiency of such a system is determined by the total turnover number (TTN). TTN is defined as the total number of moles of product formed per mole of cofactor or enzyme during the course of the entire reaction. For an efficient and economic system, generally, a cofactor regeneration method must be capable of recycling the cofactor 10^2 - 10^6 times, depending on the initial cost of the cofactor and the value of the product synthesized (Chenault and Whitesides, 1987; Donk and Zhao, 2003). The application of whole microbial cells with in situ cofactor regeneration for synthetic purpose could avoid the above-mentioned problems, but their selectivity is poor (Goubet, et al. 2002;

Grizon, et al. 2004), may involve secondary reactions and also substrate or product inhibition may occur. For these reasons, often the enzymatic methods with isolated enzymes for the cofactor regeneration is used because they are specific for the regeneration of enzymatically active cofactor and are mostly compatible with other components of an enzymatic reaction. In this way a high TTN is achieved and the cofactor is converted specifically, without causing secondary reactions. With the enzymatic method for cofactor regeneration, two general concepts are used which are:

1. Substrate-coupled regeneration: In the substrate-coupled approach, the oxidoreductase serves not only the substrate reduction but also the oxidation of the cosubstrate and therefore both the main reaction and the regeneration of the cofactor are catalyzed by the same enzyme. In the case of alcohol dehydrogenases, cheap alcohols like ethanol or 2-propanol are widely used as the cosubstrate for the regeneration system. In this process, the cosubstrate is given in surplus amount which is oxidized, and the substrate is reduced. With the oxidation of the cosubstrate, the reduced form of the cofactors is restored.

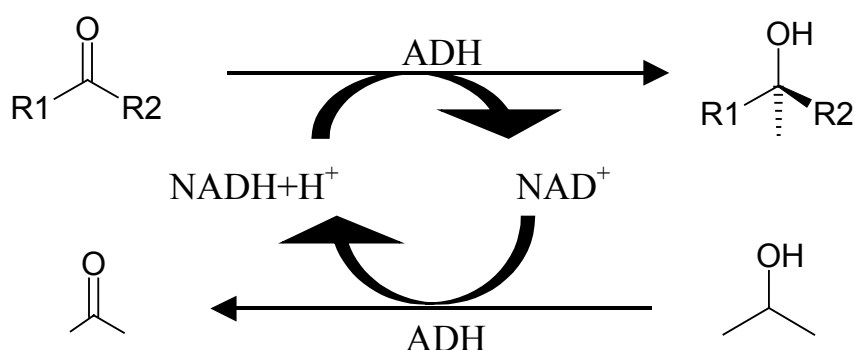


Figure 2: Substrate-coupled cofactor regeneration of alcohol dehydrogenase.

2. Enzyme-coupled regeneration: In the case of enzyme-coupled approach, the reduction of the main substrate and the cofactor regeneration reaction are catalyzed by two different enzymes. For this, the substrate of the second enzyme is added to the system. The oxidation of this substrate leads to the reduction of the cofactors. For this method, most often, the formate dehydrogenase (FDH) from *Candida boidinii* is used (Slusarczyk et al., 2000), which under the reduction of NAD^+ produces CO_2 from formate.

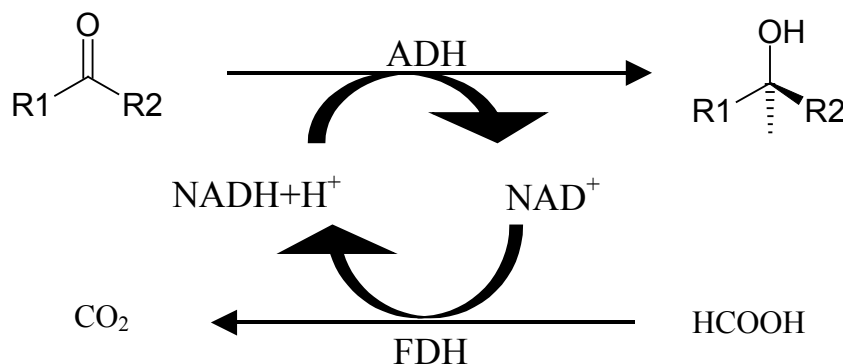


Figure 3: Enzyme-coupled cofactor regeneration of alcohol dehydrogenase.

The biochemical characteristics of CPCR and CpSADH allow both the enzyme- and substrate-coupled approaches for coenzyme regeneration (Peters et al., 1993c; Zelinski et al., 1999; Endo and Koizumi, 2001; Matsuyama et al., 2002). Due to the alcohol dehydrogenase activity of the CPCR and CpSADH, NADH regeneration may be carried out in a substrate coupled reaction using 2-propanol as hydrogen donor (Figure 2). The enzyme coupled mode can be carried out with FDH as a second enzyme and formate as the ultimate hydrogen donor (Figure 3).

1.7 Alcohol dehydrogenases in organic solvents

The application of enzymes in organic or aqueous-organic solvents (liquid-liquid two-phase system) rather than conventional media (purely aqueous system) is increasingly used. The presence of a substantial amount of organic solvent in the biocatalytic reaction medium may offer certain important advantages in comparison to the aqueous reaction medium. High concentrations of poorly water-soluble substrates and/or products are possible in organic solvent-containing media. The chance of microbial contamination is reduced because microorganisms cannot grow in organic solvents. Furthermore, reaction equilibrium may be shifted favorably, and substrate and/or product hydrolysis can be largely prevented (Brink and Tramper, 1985). In case of two-liquid-phase systems extra advantageous phenomena could occur. Substrate or product inhibition may be reduced as a consequence of a lower inhibitor concentration in the aqueous environment of the enzyme, and recovery of product and biocatalyst is facilitated (Brink and Tramper, 1985) because in such a two-phase system, the product is extracted in the organic phase while the biocatalyst remains in the aqueous phase. Stabilization of the biocatalyst, particularly thermo-stability is another advantage of organic

solvent containing media (Zaks and Klibanov, 1984) because many denaturing mechanisms are based on hydrolytic reactions. The reaction in organic solvents can be influenced by the choice of the organic solvents. Many organic solvents lead to a variation of the substrate and cause a change in the chemo-, regio- and stereo-selectivity of the biocatalyst. Contrary to the advantages, the main drawbacks of introducing an organic solvent is smaller catalytic activities, which therefore require larger quantities of biocatalyst, as well as possible denaturation of sensitive biocatalysts responsible for the desired bioconversion by the organic solvents (Faber, 2004). The influence of this denaturation could be due to the withdrawal of water from the biocatalyst in presence of organic solvents, which is required for its activity and/or catalytic active conformation. Therefore, just a hydrate covering can be sufficient for conformational flexibility needed for the catalytic activity (Zaks and Klibanov, 1988). A direct contact with the organic solvents can lead to inhibition or change in conformation of the enzyme. In case of aqueous-organic two-phase systems, the organic solvent can lead to deactivation of the enzyme due to the occurrence of liquid-liquid interface between the solvents and presence of residual amounts of organic solvent in aqueous medium (Ghatorae et al., 1994; Halling et al., 1998). To overcome this deactivation, methods to guide the choice of the proper solvent have been proposed, such as that reported by Brink and Tramper (1985). Laane et al. (1985; 1987) suggested using the polarity of solvents, expressed by the log P value (Logarithm of the partition coefficient of a given compound in the standard n -octanol/water two-phase system) as the main criterion for optimizing organic solvents in multi liquid-phase biocatalysis. But, Filho et al. (2003) reported that the log P concept is not satisfactory to guide the choice of an organic solvent serving as the second phase in ADH-catalyzed reactions and therefore recommended that instead of the hydrophobicity of the solvent (log P) its functionality should be considered when screening for a solvent.

Increasingly aqueous-organic two-phase systems are used for enzymatic synthesis with alcohol dehydrogenases (Wandrey et al., 2003; Gröger and Hummel, 2004) without intermixing the phases. Several cofactor-dependent alcohol dehydrogenases had been extensively used in such a liquid-liquid aqueous-organic two-phase system for synthetic applications and were found to have a catalytic active conformation in the presence of organic solvents. These include, YADH (Yeast alcohol dehydrogenase), HLADH (Horse liver alcohol dehydrogenase), TBADH (*Thermoanaerobium Brockii* alcohol dehydrogenase), LBADH (*Lactobacillus brevis* alcohol dehydrogenase), LmADH (*Lactobacillus minor* alcohol dehydrogenase), ReADH (*Rhodococcus erythropolis* alcohol dehydrogenase) (Deetz and

Rozzell, 1988; Guinn et al., 1991; Snijder-Lambers et al., 1991; Jönsson et al., 1999; Filho et al., 2003; Gröger et al., 2003; Gupta et al., 2004). Of the examined ADHs, the stability and activity of the HLADH both as native and as immobilisate bound to different carriers was found to be the best in organic media with different water contents and using substrate-coupled regeneration mode (Grunwald et al., 1986; Zaks and Klivanov, 1988; Deetz and Rozzell, 1988; Snijder-Lambers et al., 1991; Larsson et al., 1991; Guinn et al., 1991; Filho et al., 2003). However, a limiting factor for employing alcohol dehydrogenases in organic solvents is the necessary use of the nicotinamide adenine dinucleotide cofactors which are strictly polar molecules and thus insoluble in organic solvents. They remain therefore bound to the biocatalysts and cannot diffuse through the solvents. Thus only substrate-coupled cofactor regeneration method can be employed. The enzyme-coupled regeneration with formate dehydrogenase is also possible in aqueous-organic systems, but the FDH from *C.biodinii* is reported to be very unstable in the presence of organic solvents (Gröger et al., 2003). But a mutant of the FDH from *C.biodinii* showed higher stability in hexane (Slusarczyk et al., 2000; Gröger et al., 2003) and was thus employed in the enzyme-coupled cofactor regeneration in organic solvents.

1.8 Immobilization

Immobilized enzymes are defined as “enzymes which are physically confined or localized in a certain region of space with retention of their catalytic activities, and which can be used repeatedly and continuously” (Katchalski-Katzir, 1971). Like any other methods, the immobilization of enzymes has its own advantages and disadvantages. The main advantages include low downstream processing cost, possibility of enzyme recycling, separation of the enzyme catalyst easily from the reaction mixture, better stability especially true for ADH enzymes towards heat (Liao and Chen 2001; Julliard et al., 1986), cofactor binding to enzyme (less waste) and easy realization of continuous production. The main disadvantages of enzyme immobilization are loss of absolute enzyme activity due to the immobilization process, additional costs of carriers or other reagents used for immobilization process and potential for mass transfer limitations. However, immobilization of enzymes has become an indispensable part of the industrial biotransformations especially useful for expensive enzymes (Liese et al., 2000). The choice of the immobilization method therefore depends on the enzyme stability (Keinan et al., 1986b; Kelly et al., 1977) and the process of application. Organic solvents are frequently used as reaction medium in order to facilitate reactions with hydrophobic, non-water-soluble substrates. However, the disadvantages of employing alcohol

dehydrogenases are low enzyme stability and activity in organic solvents, since the natural environment of many enzymes including the alcohol dehydrogenases is aqueous. This leads to very fast denaturation of the enzyme by the organic solvent (Keinan et al., 1986b; Orlich, 2000; Gröger et al., 2003). On the other hand, the poor solubility of many substrates and/or products does not allow their employment in pure aqueous medium. The influence of this denaturation by the organic solvent might be reduced and on the same time the solubility of many hydrophobic substrates can be increased, and consequently the biocatalyst stability enhanced, by immobilizing the biocatalysts at or in a protective support. In that case one also benefits from the intrinsic advantages provided by immobilization itself.

There are several possibilities of immobilization of biocatalysts for the employment in organic solvents. In general, the most commonly used immobilization methods are adsorption or covalent linkage to the carrier. Adsorption is the simplest and the oldest method of immobilizing an enzyme onto a water-insoluble carrier. In this method, the biocatalysts are held to the surface of the carriers by physical forces (van der Waals forces). A disadvantage, however, of such a method is the relative weakness of adsorptive binding forces. Adsorbed biocatalysts are easily desorbed by temperature fluctuations, and even more readily by changes in substrate and ionic concentrations (Hartmeier, 1988). In covalent binding, the atoms are linked by means of shared electron pairs. This produces a tight association between a biocatalyst and a carrier and hence the biocatalyst is not desorbed from the carrier and therefore is more suited for application in non-conventional media. The covalent binding of enzymes to solid supports can effectively extend the lifetime of the biocatalysts by protecting the native three-dimensional (3D) structure of enzyme molecules and may result in enhanced enzyme activity and stability in organic solvents as compared with that of native enzyme in the same reaction media (Wang et al., 2001). There are many protocols to covalently immobilize proteins (Royer, 1980; Klivanov, 1983; Rosevear, 1984; Hartmeier, 1985; Chibata et al., 1986; Katchalski-Katzir, 1993; Bickerstaff, 1997). However, most of these protocols for covalent protein immobilization have some drawbacks when trying to quantitatively immobilize, under mild experimental conditions, large amounts of proteins per milliliter of support with a long-term handling of the activated supports, e.g., when immobilization is carried out at an industrial level. Thus, there are not so many easy-to-perform methodologies that may be applied to immobilize large amounts of any protein in any solid under mild experimental conditions.

Epoxy-activated supports seem to be almost ideal systems to develop very easy protocols for enzyme immobilization (Hartmeier, 1985; Katchalski-Katzir, 1993; Bickerstaff, 1997; Wheatley and Schmidt, 1999; Katchalski-Katsir and Kraemer, 2000). Epoxy groups are very stable at neutral pH values even in wet conditions, and hence commercial supports can be stored for long periods of time and can be prepared quite far from the place where the enzyme has to be immobilized. Moreover, these epoxy supports may be utilized to stabilize enzymes through multipoint covalent attachment by controlling the enzyme-support interactions (Mateo et al., 2000a; Mateo et al., 2002). Furthermore, epoxy supports are able to react with different nucleophilic groups on the protein surface (e.g. amino, hydroxy, or thiol moieties) to form extremely strong linkages (secondary amino bonds, ether bonds, thioether bonds) with minimal chemical modification of the protein. But, epoxy groups are hardly reactive for enzyme immobilization under mild experimental conditions (neutral pH, low ionic strength) (Hannibal-Friederich et al., 1980; Melander et al., 1984; Smalla et al., 1988; Wheatley and Schmidt, 1999). Thus, immobilization on epoxy supports proceeds via a two-step immobilization mechanism: a rapid preliminary physical adsorption, and then the covalent attachment between nucleophilic groups of the enzyme (amino, thiol, or hydroxy groups) and a high density of nearby epoxy groups in the support is strongly favored (Melander et al., 1984; Smalla et al., 1988; Wheatley and Schmidt, 1999; Mateo et al., 2000b; Mateo et al., 2002). For this reason, most of the commercial supports designed for protein immobilization have a fairly hydrophobic nature, and the recommended immobilization conditions include the use of a high ionic strength (to force the hydrophobic adsorption of the proteins). The commercially available SEPABEADS[®] EC-EP (Resindion S.r.l., Italy) is a conventional epoxy-activated support which has been used for performing very easy industrial immobilization of biocatalysts. By just immobilizing the enzymes inside porous supports, it may be possible to increase the enzyme operational stability by preventing any intermolecular process (proteolysis, aggregation) and by preserving the enzyme from interactions with external interfaces (air, oxygen, immiscible organic solvents, etc.) (Gupta, 1991; Bes et al., 1995; Fernandez-Lafuente et al., 1998). However, random immobilization might not promote any conformational stabilization of immobilized enzymes. In fact, there are reports of immobilization procedures with no effect or even negative effects on the stability of some enzymes (Klibanov, 1982; Rody et al., 1983; Gianfreda et al., 1991; Nanalov et al., 1993; Desmukh et al., 1993). However, it is generally accepted that such stabilization should be achieved if the immobilization of each enzyme molecule occurs through several residues, mainly if the reactive groups in the support are secluded from its surface through very short

spacer arms. In this way, all the residues of the enzyme molecule involved in immobilization have to preserve their relative positions almost completely unaffected during any conformational change promoted by heat, organic solvents, or any other distorting agents (Guisan, 1988; Mozhaev et al., 1990; Guisan et al., 1991). Thus, such multipoint covalently immobilized enzymes should become much more stable than their soluble counterparts or than randomly immobilized derivatives. The Sepabeads EC-EP is not only capable of immobilizing enzymes but also of achieving multipoint covalent attachment between the immobilized enzyme and the support. However, the hydrophobicity of the support may create some stability problems on the immobilized enzyme (Mateo et al., 2002). Having in mind this two-step mechanism for the covalent immobilization of proteins on epoxy supports, multifunctional supports have been recently proposed as a second generation of activated supports that are able to covalently immobilize enzymes, antibodies, and other proteins under very mild experimental conditions (Mateo et al., 2000b; Mateo et al., 2001; Abad et al., 2002; Mateo et al., 2003). In general, these multifunctional supports should contain two types of functional groups: (1) groups that are able to promote the physical adsorption of proteins (e.g., by ionic exchange, by adsorption on immobilized metal chelates); and (2) groups that are able to covalently immobilize the enzyme (e.g., epoxy groups). In this way, enzymes are physically adsorbed on the support first via different phenomena and then some covalent linking could take place between nucleophilic groups of the protein (amino, thiol, and hydroxyl ones) and the epoxy groups on the support surface. The commercially available SEPABEADS[®] EC-HFA (support activated with epoxy-amino groups) (Resindion S.r.l., Italy) is an example of such a hetero (multi) functional support which is implied by the introduction of a modification of the epoxy groups on the support surface (e.g., through introduction of amino groups) and therefore is an alternative to conventional epoxy supports in the immobilization of industrial enzymes. In this case, the epoxy moieties have been introduced by modification of a layer of ethylenediamine covalently bound to the support surface. Therefore, using these supports, there is a 1:1 ratio between the number of amino groups and the reactive epoxy groups (that determine the physical adsorption and covalent immobilization rate, respectively). Thus, this support has a great anionic exchanger power and a high number of epoxy groups. Immobilization proceeds at low ionic strength using amino-epoxy Sepabeads while requiring high ionic strength using conventional monofunctional epoxy supports (Mateo et al., 2003; Torres et al., 2003).

Another well-known method of immobilization of enzymes for their application in hydrophobic solvents is via entrapment in hydrogels. An increase in the stability and also the activity of the alcohol dehydrogenase can be achieved in hydrophobic media by such an immobilization. In a hydrogel, the enzyme remains in its natural aqueous environment. The hydrogels protect the enzyme against the influence of organic solvents, but at the same time, reactions with poorly water-soluble substrates to produce single enantiomer are made possible (Ansorge-Schumacher et al., 2000). Hydrogels are characterized by a high water-content and result mostly from the cross-linking of hydrophilic monomers or also polymers that form a lattice structure/network into which biocatalyst molecules can be entrapped. They can be used in organic solvents as the so-called gel-stabilized two-phase system. The surrounding solvent serves thereby as reservoir for hydrophobic substrates, which can diffuse into the matrix, while the product is extracted directly from the gel. For the entrapment of enzymes both artificial and natural matrices are suitable. Natural materials are non-toxic and the gelation process is usually very gentle. The simplest methods for gel formation are ionotropic and cooling gelation. The most common method for the production of immobilisate by ionotropic gelation is introducing the solution in drops, which can be gelled into an aqueous ionic-bath. However, this method has a disadvantage with cofactor-dependent enzymes in that it leads to very quick washing away of the cofactor (König, 1996). Polyvinyl alcohol is an example of a more modern synthetic polymer, which forms a hydrogel by cooling gelation. It is non-toxic and therefore also suitable for sensitive enzymes. Gelation of the aqueous polyvinyl alcohol solutions takes place by “freezing and thawing” mechanism, in which the solution is first frozen and then slowly thawed. Hardening of the gels is based on the formation of hydrogen bonds between the polymer chains (Lozinsky and Plieva, 1998; Prüsse, 2000), between which biocatalyst and cofactor can be entrapped. This method is particularly very suitable for sensitive enzymes, since no higher temperatures than ambient temperature are needed and the biocatalyst does not come in contact with toxic monomers, as it is for example with the production of polyacrylamide gels.

1.9 Aim and motivation of the research work

Chiral compounds are valuable building blocks for the synthesis of pharmaceuticals, agrochemicals and natural materials. For the synthesis of these products, oxidoreductases are of special interest, because of high enantioselectivity and a wide substrate spectrum.

The chiral hydroxyacids and/or esters of today always resulted mainly through microbial reductions with Baker's yeast. The ADH1 reacts mainly with aldehydes to produce the *S*-alcohols and its substrate spectrum is also limited. In addition, instability to heat and organic solvents are also exhibited. Other well-known carbonyl reductases need NADPH as cofactor and are therefore cost-intensive for industrial use.

The CPCR (Peters et al., 1992, 1993a, Kula et al., 1996) is a very versatile biocatalyst and the industrial use of this enzyme is of large interest. But the fermentation of the wild type of *C.parapsilosis* resulted only in small quantities of the enzyme (< 0.03% of the total cell proteins). So for industrial application of this enzyme, sufficient and economic availability of the biocatalyst must be guaranteed.

Another well-known industrially important secondary alcohol dehydrogenase is the CpSADH (Kojima et al., 1995). Both the CPCR and the CpSADH are two oxidoreductases from two different types of *C.parapsilosis* strains. Both these enzymes are NADH-cofactor dependent, exhibit a wide substrate spectrum and are *S*-specific. However, it was not clear which of the two oxidoreductases is the real CPCR with its broad versatility and substrate spectrum as has been described in literature data. Furthermore, old literature data available for the CPCR showed that the enzyme had totally different biochemical characteristic features than that of CpSADH. But, recent characterization of the commercially available purified wtCPCR (Juelich Fine Chemicals, Juelich, Germany) by Steinsiek (2006) showed that certain specific biochemical characteristics like the pH- and temperature-optimum data of the CPCR indeed agreed much more with the data of the CpSADH though the range and percentual conversion of significant substrates matched with the results described by Peters et al. (1993a).

The present work would be to identify which of the two oxidoreductases that are found in the *C.parapsilosis* is the real CPCR. With this view, both the genes coding for CPCR and CpSADH would be amplified by PCR from the gDNA of the “CPCR” type strain of *C.parapsilosis*, after which conditions would be created for the employment of both these enzymes within the technical range by cloning and expression of the CPCR and the CpSADH in a suitable expression system, preferably *E.coli*. For amplification of the CPCR gene, the method of reverse genetics was selected. From the partial N- and C-terminal protein sequences (Bönitz, 2001) of the enzyme, the DNA sequence would be derived by the method of PCR based genome walking (Siebert et al., 1995). For the amplification of the CpSADH gene, primers would be deduced from the published sequences and via conventional PCR, the DNA sequence would be derived. Both the recombinant enzymes would then be characterized with respect to biochemical properties, molecular features and substrate spectrum and compared with the wild type of CPCR. In addition, the recombinant protein would be made available for easy purification using different optimized chromatographic methods. Furthermore, attempts would be made for downstream application of the recombinant enzyme in conventional and non-conventional media (organic solvents) using both soluble and immobilized enzyme. The enzymatic synthesis in organic solvents is particularly of interest due to the possibility of stereospecific reductions of industrially important hydrophobic substrates like acetophenone and its derivatives. Immobilization of the recombinant enzyme would be carried out via entrapment in hydrogels and covalent binding and the immobilisate would be employed for synthesis in organic solvents which serves as the substrate reservoir. The stability and activity of the soluble enzyme (in pure aqueous and in aqueous-organic system) would then be compared with the immobilisate in an aqueous-organic two-phase system.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Agar	Beco (Nuernberg, Germany)
Acrylamide-Bisacrylamide-Solution	MP Biomedicals (Aurora, USA)
BIO-RAD Protein Assay	Bio-Rad Laboratories GmbH (Munich, Germany)
DAB-substrate	Roche Applied Science (Mannheim, Germany)
Glucose-Monohydrate	Roquette GmbH (Frankfurt, Germany)
Glycogen	Serva (Heidelberg, Germany)
Isopropyl- β -D-thiogalactopyranoside	Qbiogene (Heidelberg, Germany)
Siliconoil	Drawin (Wacker-Chemie, Munich Germany)
SeaKem LE Agarose	FMC BioProducts (Rockland, USA)

All other chemicals not mentioned here were of high technical grade and obtained from Sigma (Steinheim, Germany), Aldrich (Taufkirchen, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany). Growth media components were bought from Difco (Detroit, USA), Ohly (Hamburg, Germany) or Roth (Karlsruhe, Germany). All substrates for the carbonyl reductase were obtained from Aldrich (Taufkirchen, Germany), Fluka (Neu-Ulm, Germany), Lancaster (Frankfurt a. M., Germany) or Merck (Darmstadt, Germany) and were of the highest quality.

Cofactors

NAD⁺ (β - nicotinamide adenine dinucleotide; oxidized form) was purchased from Fluka (Neu Ulm, Germany). The reduced form of the cofactor NADH + H⁺ (β -nicotinamide adenine dinucleotide; Reduced di-sodium salt; > 99.5%), was obtained from Juelich Fine Chemicals (Juelich, Germany).

2.1.2 Devices

Agarose gel electrophoresis chamber	Bio-Rad (Munich, Germany)
Centrifuge (Sorvale® RC5B-Refrigerated Superspeed Centrifuges)	DuPont Instruments (Bad Hamburg, Germany)
Chamber for Western blot-Analysis (Xcell SureLock™ Mini-Cell with XCell II™ Blot Module)	Invitrogen (Karlsruhe)
FPLC (LKB-Controller, LCC-501 Plus)	Pharmacia (Uppsala, Sweden)
Gas chromatograph (HP 5890 Series II)	Hewlett Packard (Walbronn, Germany)
HPLC	Beckmann Coulter System Gold (Munich, Germany)
Incubation shaker (Multitron II)	Infors AG (Bottmingen, Switzerland)
Lab shaker	IKA®-Werke GmbH & CO. KG (Staufen)
Lyophilizer	Schrader (Friedland, Germany)
Microplate Reader Spectramax Plus 340	Molecular Devices (Munich, Germany)
Microscope DM RB	Leitz (Wetzlar, Germany)
Over-head-shaker (RK 10-VS)	Heto Lab Equipment (Allerød, Denmark)
Protein gel chamber (Mini Protean II)	Bio-Rad Laboratories (Munich, Germany)
Spectrophotometer DU®Series	Beckmann (Fullerton, CA)
Table centrifuge (GS-15R-Zentrifuge)	Beckmann (Munich, Germany)
Table centrifuge (Minispin Plus)	Eppendorf (Hamburg, Germany)
Thermoblock (Thermomixer 5436)	Eppendorf (Hamburg, Germany)
Thermo-PCR-Cycler GeneAmp® PCR System 9700	Applied Biosystems (Foster City, USA)
Ultrasonication device (Sonopuls HD200 with Micro tip MS73)	Bandelin Electronic (Berlin, Germany)
UV- Transilluminator Gel Doc 2000™	Bio-Rad Laboratories (Munich, Germany)
Vivaspin-Concentrator 20 ml	Vivascience, Sartorius group (Goettingen, Germany)
Vortex (REAX 2000)	Heidolph Instruments (Schwabach, Germany)

Chromatography matrices

Anion Exchange: Macro Prep High Q	Bio-Rad (Munich, Germany)
IMAC: Ni-NTA Superflow and Ni-NTA Agarose	Qiagen GmbH (Hilden, Germany)

2.1.3 Cultivation media

All media for cell cultivation namely LB-medium and TB-medium were based on the standard protocols (Sambrook et al., 1989). The protocol for yeast medium was as described by DSMZ, Germany. If a percentage is given for a component in media or buffer recipes (e.g. 10% NaCl), it describes a weight per volume ratio. Solidified agar plates were made with 2% agar.

LB medium

Tryptone	1%
NaCl	1%
Yeast extract	0.5%

Deionized water as required. The pH was adjusted to 7.2 with NaOH. The solution was autoclaved for 20 minutes at 121 °C.

TB medium

Deionized water, to 900 ml	
Glycerol	4 ml
Tryptone	12 g
Yeast extract	24 g

The components were shaken until the solutes had dissolved and then sterilized by autoclaving for 20 minutes at 121 °C. The solution was allowed to cool to 60 °C or less, and then 100 ml of a sterile solution of 0.17 M KH_2PO_4 , 0.72 M K_2HPO_4 was added. (This solution was made by dissolving 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 ml of deionized H_2O . After the salts had dissolved, the volume of the solution was adjusted to 100 ml with deionized H_2O and sterilized by autoclaving for 20 minutes at 121 °C.)

Yeast medium

Yeast extract	0.3%
Malt extract	0.3%
Peptone	0.5%
Glucose	1%

Deionized water as required. The solution was autoclaved for 20 minutes at 121 °C.

SOC medium (100 ml)

Tryptone	2 g
Yeast extract	0.5 g
1 M NaCl	1 ml
1 M KCl	0.25 ml
2 M Mg ²⁺ stock, (filter-sterilized)	1 ml
2 M glucose, (filter-sterilized)	1 ml

Tryptone, yeast extract, NaCl and KCl were added to 97 ml deionized water and stirred to dissolve. This was then autoclaved for 20 min at 121 °C and cooled down to room temperature. 2 M Mg²⁺ stock and 2 M glucose were then added, each to a final concentration of 20 mM. The final volume of the solution was then brought to 100 ml with sterile, deionized water.

2 M Mg²⁺ stock

MgCl ₂ ·6H ₂ O	20.33 g
MgSO ₄ ·7H ₂ O	24.65 g

Deionized water was added to 100 ml and the solution was filter sterilized.

Stock solutions for antibiotics

Ampicillin	100 mg/ml in deionized water. Filter sterilized through 0.2 µm filter membranes. Stored at –20 °C. Used at 100 µg/ml.
Kanamycin	30 mg/ml in deionized water. Filter sterilized through 0.2 µm filter membranes. Stored at –20 °C. Used at 30 µg/ml.

2.1.4 Microorganisms

Candida parapsilosis DSMZ 70125

<i>E. coli</i> DH5 α	<i>[(supE44 ΔlacU169 Φ80 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1] (Pharmacia)</i>
<i>E. coli</i> JM109	<i>[recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ (lac-proAB) F'(traD36 proAB⁺ lacI^q lacZΔM15)] (Stratagene)</i>
EZ competent cells	<i>[F':Tn10(Tc^r) proA⁺B⁺ lacI^qZΔM15] recA1 end A1 recA1 end A1 hsdR17 (<i>r</i>_{K12}⁻ <i>m</i>_{K12}⁺) lac glnV44 thi-1 gyrA96 relA1 (Qiagen)</i>
<i>E. coli</i> BL21(DE3)	<i>[B F ompT hsdS_B (<i>r</i>_B⁻ <i>m</i>_B⁻) gal dcm (DE3)] (Novagen)</i>

2.1.5 Vectors

pKK223-3	4584 bp, ColE1-Replicon, <i>P</i> _{lac} , Amp ^R (Pharmacia)
pDrive	3851 bp, ColE1-Replicon, LacZ α -peptide, <i>P</i> _{T7} , <i>P</i> _{SP6} Amp ^R , Kan ^R (Qiagen)
pET-26b(+)	5360 bp, ColE1-Replicon, <i>P</i> _{T7lac} , Kan ^R (Novagen)

2.1.6 Materials for Molecular biology work

Antibody- conjugate Anti-His ₆ -Peroxidase	Roche Applied Science (Mannheim, Germany)
Advantage2 Polymerase Mix	BD Biosciences (Heidelberg, Germany)
Calf Intestine Alkaline Phosphatase	MBI Fermentas (St. Leon-Rot, Germany)
Eppendorf [®] HotmasterMix	Eppendorf (Hamburg, Germany)
GeneRuler 1 kb DNA Ladder	MBI Fermentas (St. Leon-Rot, Germany)
Isis-& Go [™] Mastermix	Qbiogene (Heidelberg, Germany)
PageRuler [™] Prestained Protein Ladder	MBI Fermentas (St. Leon-Rot, Germany)
PageBlue [™] Protein Staining Solution	MBI Fermentas (St. Leon-Rot, Germany)
PVDF Membrane	Invitrogen (Karlsruhe, Germany)
Restriction Enzymes	MBI Fermentas (St. Leon-Rot, Germany)

T4 DNA Ligase	MBI Fermentas (St. Leon-Rot, Germany)
TripleMaster [®] PCR System	Eppendorf (Hamburg, Germany)
<u>Kits</u>	
BD Universal GenomeWalker Kit	BD Biosciences, Clontech (Heidelberg, Germany)
E.Z.N.A. Gel Extraction Kit	PEQLAB (Erlangen, Germany)
E.Z.N.A. Plasmid Miniprep Kit I	PEQLAB (Erlangen, Germany)
PCR Cloning Plus Kit	Qiagen (Hilden, Germany)
PCR Purification Kit	Qiagen (Hilden, Germany)

The above-mentioned materials were used according to the manufacture's instructions.

2.2 Microbiological methods

2.2.1 Conservation and storage of microbiological strains

For long-term storage of *E.coli* cells, cryo-cultures were made either with DMSO or with glycerol.

DMSO

A single colony was picked up from LB-agar plate and inoculated into a 5 ml liquid LB medium containing appropriate antibiotic if required, incubated at 37 °C on a shaker until the OD₆₆₀ reached 0.8. This culture was transferred to cryo-vials and then 5% (v/v) of sterile DMSO was added. The cells were shock-frozen in liquid nitrogen and stored at –80 °C.

Glycerol

This method was used for preparing stock cultures of *E.coli* cells harboring either pET vectors or pET recombinants. For this, a single colony was picked up from LB-agar plate and inoculated into a 5 ml liquid LB medium containing appropriate antibiotic if required, incubated at 37 °C on a shaker with vigorous shaking until the OD₆₀₀ reached 0.6-0.8. Then 0.9 ml of the culture was removed and transferred to a cryo-vial, and 0.1 ml of 87% glycerol was added. The culture was mixed well and stored at –80 °C.

2.2.2 Cell cultivation

Cell cultivation was carried out either in shake-flasks with baffles or in solidified agar plates with the media composition as described in section (2.1.3).

Candida parapsilosis

The cells were grown overnight either in liquid yeast medium at 37 °C in a shaker incubator at 200-250 rpm, or on agar-solidified (2%) yeast-medium plates.

Escherichia coli

E.coli strains were grown in LB or TB medium at 37 °C, 140-200 rpm, in a volume ranging from 10-1000 ml in shake flasks with baffles by inoculating one colony from agar plate or with preculture in a ratio of 1:25 or 1:50 (4-2% v/v). The ratio between the volume of the media and the volume of the shake flask was 1:5 (e.g. 100 ml media was used for cultivation in a shake flask having a volume of 500 ml). For isolation of vector material or for screening of clones, *E.coli* strains harboring plasmids were grown overnight at 37 °C in a shaker incubator at 140-160 rpm containing necessary antibiotic in the media composition.

Expression of recombinant CpSADH and recombinant CPCR in *E.coli*

The expression of carbonyl reductase was carried out in two different types of *E.coli* strains namely, JM109 and BL21(DE3). The recombinant constructs JM109pKK223-3CpSADH18 (containing the gene for CpSADH) and JM109pKK223-3CPCR9 (containing the gene for CPCR) was constructed without His₆-tag on the gene of interest. The other set of recombinant constructs BL21(DE3)pET26b+CpSADH28his (containing the gene for CpSADH) and BL21(DE3)pET26b+CPCR9his (containing the gene for CPCR) was constructed with His₆-tag on the N-terminal of the gene of interest.

Without His₆-tag:

The *E.coli* strain JM109, containing the recombinant CpSADH and CPCR gene was grown overnight in 10-200 ml TB_{amp} or LB_{amp} medium at 37 °C, 140 rpm. This overnight preculture was then added to 100-4000 ml of the main culture in a ratio of 1:25 or 1:50 (preculture: main culture) and grown at 30 °C, 100-120 rpm, until the OD₅₅₀ reached 1.2-1.6 (between early and mid-log phase). At this point the induction for protein expression was carried out by the addition of 1 mM IPTG and the production strains were cultivated for an additional 6 hours also at 30 °C and 100-120 rpm. After the induction phase, the cells were harvested by

centrifugation at 20000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellets were washed with ice-cold 20 mM TEA-HCl (pH 8) and re-centrifuged with the same conditions as stated above. The supernatant was again discarded and the pellets were stored at –20 °C till use.

With His₆-tag:

The *E.coli* strain BL21(DE3), containing the recombinant CpSADH and CPCR gene with an N-terminal His₆-tags was grown overnight in 10-200 ml TB_{kan} medium at 37 °C, 140 rpm. This overnight preculture was then added to 100-4000 ml of the main culture (TB_{kan} medium) in a ratio of 1:50 (preculture: main culture) and grown at 30 °C, 100-120 rpm, until the OD₆₀₀ reached 0.6-0.8. At this point the induction for protein expression was carried out by the addition of 1 mM IPTG and the production strains were cultivated for an additional 6 hours at 28 °C and 100-120 rpm. After the induction phase, the cells were harvested by centrifugation at 20000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellets were washed with ice-cold 20 mM Tris-HCl (pH 8) and re-centrifuged with the same conditions as stated above. The supernatant was again discarded and the pellets were stored at –20 °C till use.

2.2.3 Cell disruption and preparation of cleared *E.coli* lysates under native conditions

E.coli cells were disrupted by ultra-sonication on ice using a sonicator equipped with a microtip (Sonopuls HD200).

Lysis of recombinant constructs JM109pKK223-3CpSADH18 and JM109pKK223-3CPCR9

The frozen pellets were thawed on ice for 15 min and resuspended with Lysis Buffer I at 4 ml per gram wet weight of cells. Lysozyme was added to a final concentration of 1 mg/ml and the cell suspension was thereby incubated on ice for 30-40 min. Sonication was carried out with sonicator using four 1 min bursts with 70% intensity and with a 1 min cooling period between each burst. The lysate was centrifuged at 20000 rpm for 20-30 min at 4 °C to pellet the cellular debris. The supernatant was stored and used as cell free cleared lysate (crude extract) for further experiments e.g. protein purification (2.4.3) or activity tests (2.4.2).

Lysis Buffer I: 50 mM as well as 100 mM TEA-HCl (pH: 8.3 as well as 7.5).

Lysis of recombinant constructs BL21(DE3)pET26b+CpSADH28his and BL21(DE3)pET26b+CPCR9his

The frozen pellets were thawed on ice for 15 min and resuspended with Lysis Buffer II at 4 ml per gram wet weight of cells. Lysozyme was added to a final concentration of 1 mg/ml and the cell suspension was thereby incubated on ice for 30-40 min. Sonication was carried out with sonicator using six 30 sec bursts with 70% intensity and with a 1 min cooling period between each burst. The lysate was centrifuged at 20000 rpm for 20-30 min at 4 °C to pellet the cellular debris. The supernatant was stored and used as cell free cleared lysate (crude extract) for further experiments e.g. protein purification (2.4.3) or activity tests (2.4.2).

Lysis Buffer II (1 liter):

50 mM NaH ₂ PO ₄	6.90 g NaH ₂ PO ₄ ·H ₂ O (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
10 mM imidazole	0.68 g imidazole (MW 68.08 g/mol)

The pH was adjusted to 8.0 using NaOH.

2.2.4 Preparation of competent cells of *E.coli*

Competent cells of the *E.coli* strains (2.1.4) were made by the methods of RbCl (Hanahan, 1983) and CaCl₂ (Cohen et al., 1972).

Preparation according to Hanahan (1983):

One single colony of *E.coli* strain was removed and inoculated in 10 ml of LB-medium. The *E.coli* cells were grown overnight at 37 °C in a shaker incubator at 140 rpm. 1 ml of the overnight culture was added to 100 ml of prewarmed LB-broth and the culture was shaken at 37 °C and 200 rpm until an OD₆₀₀ of 0.5 was reached (approximately 90-120 min). The culture was then cooled on ice for 5 min and transferred to a sterile centrifuge tube. The cells were collected by centrifugation at low speed (5 min, 4000 x g, 4 °C). The supernatant was removed carefully and the cells were always kept on ice. The cells were resuspended gently in cold (4 °C) TfbI solution (30 ml for a 100 ml culture) and the suspension was kept on ice for an additional 15 min. The cells were thereby collected by centrifugation (5 min, 4000 x g, 4 °C). The supernatant was carefully removed always keeping the cells on ice. The cells were then gently resuspended in 4 ml of ice-cold TfbII solution. Aliquots of 200 µl of the cell suspension were prepared in sterile microcentrifuge tubes and shock-frozen in liquid nitrogen. The competent cells were stored at -80 °C.

TfBI solution (200 ml)

100 mM RbCl	2.42 g
50 mM MnCl ₂ ·4H ₂ O	2.98 g
30 mM K-acetate	0.59 g
10 mM CaCl ₂ ·2H ₂ O	0.29 g
15% glycerol	36.9 g
H ₂ O	added up to 200 ml

TfBII solution (100 ml)

10 mM RbCl	0.12 g
10 mM MOPS	0.21 g
75 mM CaCl ₂ ·2H ₂ O	1.10 g
15% glycerol	18.50 g
H ₂ O	added up to 100 ml

For TfBI, the pH was adjusted to 5.8 with acetic acid whereas for TfBII the pH was adjusted to 6.8 with 1 M NaOH. Each of the solutions was filter-sterilized with 0.2 µm diameter filters and stored at 4 °C till use.

Preparation according to Cohen (1972):

One single colony of *E.coli* strain was removed and inoculated in 5 ml of LB-medium. The *E.coli* cells were grown overnight at 37 °C in a shaker incubator at 250 rpm. 700 µl of the overnight culture was added to 100 ml of prewarmed LB-broth in a sterile 1-L shake-flask with baffles, and the culture was shaken at 37 °C and 250 rpm until an OD₅₉₀ of 0.4-0.5 was reached. Aliquots of the culture were then prepared in two 50-ml pre-chilled, sterile polypropylene tubes and incubated on ice for 5-10 min. The cells were kept cold for all the steps. Cells were collected by centrifugation for 7 min at 1600 x g and 4 °C. The supernatant was carefully removed and each of the pellets was resuspended very gently in 10 ml of ice-cold CaCl₂ solution. The cells were collected by centrifugation for 5 min at 1100 x g and 4 °C. The supernatant was carefully removed and each of the pellets was resuspended very gently again in 10 ml of ice-cold CaCl₂ solution. The resuspended cells were then kept on ice for 30 min. The cells were collected by centrifugation for 5 min at 1100 x g and 4 °C. The supernatant was carefully removed and each of the pellets was finally resuspended gently but very well in 2 ml of ice-cold CaCl₂ solution. The cells were dispensed in 200 µl volume into prechilled, sterile microcentrifuge tubes and immediately frozen at -80 °C.

CaCl₂ solution

CaCl ₂	100 mM
Glycerol	15%

The genomic DNA libraries of *C.parapsilosis* were prepared as described in the user's manual of the Universal Genome Walker kit (BD Biosciences, Clontech). Four libraries were constructed with the genomic DNA of *C.parapsilosis* by digesting with four different restriction enzymes (Blunt-end cutters) included in the kit namely, EcoRV, DraI, StuI and PvuII. The kit also provides human genomic DNA as positive control library that has to be

digested with PvuII, and a pre-constructed human genome-walker library. Each of the digested DNA libraries was ligated to the genome walker adaptor. The digested and ligated libraries were stored at -20°C till use. For long-term storage the libraries can also be stored at -80°C .

2.3.3 Synthetic DNA-oligonucleotides

As a guideline for oligonucleotide primers, the T_m -value, that is the temperature in which 50% of the strands are denatured, was determined as described below. As a starting point, an annealing temperature at least 5°C below T_m was chosen. All the primers were always constructed in the $5'$ - $3'$ direction and were synthesized from Metabion (Martinsried, Germany) or from Biomers (Ulm, Germany) at the highest purity (HPLC grade) having a concentration of $100\text{ pmol}/\mu\text{l}$.

$$T_m = 2^{\circ}\text{C} \times (\text{A}+\text{T}) + 4^{\circ}\text{C} \times (\text{G}+\text{C})$$

For genome walking:

Two gene-specific primers - one for primary PCR (GSP1) and one for nested PCR (GSP2) were designed taking a few aspects in consideration: The nested PCR primer should anneal to sequences beyond the $3'$ end of the primary PCR primer (i.e. upstream of the primary PCR primer when walking upstream and downstream of the primary PCR primer when walking downstream). The primers were constructed with at least 30 nucleotides in length and with a G/C-content of 40-60% taking into account the partial amino acids sequences of the wtCPCR.

SET A: $(3' - \text{TCG-TTA-TCG-CAT-AAC-TTA-TCG-GTT-ACT-TGG-}5')$

Primer CPCR-R1b: $5' - \text{AGC-AAT-AGC-GTA-TTG-AAT-AGC-CAA-TGA-ACC-}3'$

(For primary PCR) $(3' - \text{GTT-TGG-TGG-TGG-TCG-TGG-ACT-TTA-TGT-TTG-}5')$

Primer CPCR-R2b: $5' - \text{CAA-ACC-ACC-ACC-AGC-ACC-TGA-AAT-ACA-AAC-}3'$

(For nested PCR)

SET B:

Primer CPCR-V1: $5' - \text{ATA-ACA-ATA-TAA-ATA-GAC-CAC-GAG-GGA-ATG-}3'$

(For primary PCR)

Primer CPCR-V2: $5' - \text{ATG-CCA-ATT-GGC-TCA-ACA-CAT-TTC-TTT-CAT-}3'$

(For nested PCR)

The primers of SET B were designed based on the amplified PCR product with the primers of SET A.

For gene specific amplifications:

The forward and the reverse primers for the amplification of the CpSADH gene from *C.parapsilosis* DSMZ 70125 strain were derived from the nucleotide sequence coding for the same gene from *C.parapsilosis* IFO 1396 (Kojima et al., 1995). The forward and the reverse primers for the amplification of the CPCR gene were based on the nucleotide sequence of the PCR product that was generated with the primer combinations of SET B and adaptor primers. The list of primers is as follows:

ADH-J1	5'-CCG AAT TCC ATG TCA ATT CCA TCA AGC CAG-3'
ADH-J2	5'-CCC AAG CTT CTA TGG ATT AAA AAC AAC TCT-3'
CPCR-F1A	5'-CCG AAT TCC ATG CCA ATT GGC TCA ACA CAT-3'
CPCR-F2	5'-CCC CTG CAG TTA CTT GCT GGT GTC AAG TAC-3'
JADH-NHis	5'-CCC CAT ATG CAC CAT CAT CAT CAC CAC ATG TCA ATT CCA TCA AGC CAG-3'
JADH-CXho	5'-CCC CTC GAG CTA TGG ATT AAA AAC AAC TCT-3'
CPCR-Nhis	5'-CCC CAT ATG CAC CAT CAT CAT CAC CAC ATG CCA ATT GGC TCA ACA CAT-3'
CPCR-CXho	5'-CCC CTC GAG TTA CTT GCT GGT GTC AAG TAC-3'

2.3.4 Polymerase chain reaction (PCR)

Specific DNA fragments can be amplified *in vitro* with the help of PCR (Mullis and Faloona, 1987). Through the different cycles of PCR namely denaturation, annealing of the primers and elongation (extension) of DNA, it is possible to have an exponential amplification of the DNA template as because every newly built DNA fragment serves as a starting material for the following reaction. In the reactions, gDNA (2.3.1) or constructed libraries (2.3.2) were used as templates for the specific primers. The DNA fragments were amplified with thermostable polymerases. For preparative methods such as cloning of DNA fragments, all the PCR products were purified with the PCR Purification kit (Qiagen).

PCR set-up for the amplification of the constructed libraries with the Genome Walker kit using adaptor primers:

PCR technique was used for walking the gene and amplification of the adaptor ligated gDNA libraries of *C.parapsilosis*. Two PCR reactions followed: Primary PCR and secondary or nested PCR. Two primers were used in each PCR reaction: Adaptor primer 1 (AP1) and gene-specific primer (GSP1) for the primary PCR, while adaptor primer 2 (AP2) and gene-specific primer (GSP2) for the nested PCR.

The polymerase used was Advantage 2 Polymerase Mix (BD Biosciences). This mix contains a mixture of two different polymerases. The simultaneous use of two different DNA polymerases (primary and proofreading) in a PCR reaction allows amplification of significantly longer fragments in a process known as long and accurate PCR or long-distance PCR (LD PCR) (Barnes, 1994; Cheng et al., 1994). However, the usefulness of two-enzyme systems is not limited to LD PCR. In fact, the efficiency of most PCR reactions can be significantly improved by using the two-enzyme combination. Advantage 2 Polymerase Mix contains the *Taq* polymerase and a minor amount of proofreading polymerase. The inclusion of a minor amount of a proofreading polymerase results in an error rate that is 3-fold lower than that of conventional PCR with *Taq* alone (Barnes, 1994; Frey et al., 1995; Nelson et al., 1995). This Mix also contains TaqStart antibody, which has been shown to significantly improve the efficiency and specificity of PCR amplifications by reducing background DNA synthesis (Kellogg et al., 1994). This polymerase leaves a single 3'A-overhang on the amplified PCR product and therefore the product can be cloned into any vector having a T- or U-overhang.

The gene-specific primers used were either from SET A or from SET B (see 2.3.3). Both the adaptor primer 1 (AP1) and adaptor primer 2 (AP2) were supplied with the kit. The PCR reactions were set up as described in the user's manual of the Genome Walker kit. A "Touchdown (TD)" PCR was followed in order to get a more precise amplified PCR product without any background and to get rid of unspecific products (Hecker and Roux, 1996; Roux, 1995; Don et al., 1991).

The cyclic parameters for the primary and nested touchdown PCR were as follows:

Primary TD PCR			Nested TD PCR		
94 °C	2 sec	7 cycles	94 °C	2 sec	5 cycles
72 °C	3 min		72 °C	3 min	
94 °C	2 sec	5 cycles	94 °C	2 sec	5 cycles
70 °C	3 min		70 °C	3 min	
94 °C	2 sec	32 cycles	94 °C	2 sec	19 cycles
67 °C	3 min		67 °C	3 min	
67 °C for an additional 4 min			67 °C for an additional 4 min		
4 °C	cooling	∞	4 °C	cooling	∞

PCR set-up for the amplification of CpSADH and CPCR without His₆-tag:

For amplification of both the genes, the DraI digested library of *C.parapsilosis* was used as a template. Isis & GoTM Mastermix was used as the source of DNA polymerase. ISIS DNA polymerase is a highly accurate, thermostable DNA polymerase. It is a recombinant enzyme, originally isolated from *Pyrococcus abyssi* Orsay strain (Dietrich et al., 2002; Gueguen et al., 2001). For the amplification of the CpSADH gene, the primer combination of ADH-J1 (forward) and ADH-J2 (reverse) were used (see 2.3.3). For the amplification of CPCR gene, the primer combination of CPCR-F1A (forward) and CPCR-F2 (reverse) were used (see 2.3.3). The PCR reaction set-up for both the genes was the same, which is as follows:

PCR reaction set-up:

<u>Components</u>	<u>Volume</u>	<u>Concentration</u>
Isis & Go TM Mastermix (5 x)	10 µl	1 x
Dra I digested library	2 µl	0.6 ng
Forward primer (2 pmole/µl)	12 µl	24 pmole
Reverse primer (2 pmole/µl)	12 µl	24 pmole
Sterile deionized H ₂ O	14 µl	--
Total volume	50 µl	

The PCR cyclic parameters for amplification of CpSADH and CPCR were:

<u>Steps</u>	<u>CpSADH</u>	<u>CPCR</u>	<u>Time</u>	<u>Cycles</u>
Denaturation	93 °C	93 °C	5 min	1
Denaturation	93 °C	93 °C	30 sec	30
Annealing	50 °C	55 °C	30 sec	
Elongation	72 °C	72 °C	2 min	
Cooling	4 °C	4 °C	∞	--

PCR set-up for the amplification of CpSADH and CPCR with His₆-tag:

For amplification of both the genes, the DraI digested library of *C.parapsilosis* was used as a template. For the amplification of the CpSADH gene with His₆-tag on the N-terminal, the primer combination of JADH-NHis (forward) and JADH-CXho (reverse) were used (see 2.3.3). For the amplification of CPCR gene with His₆-tag on the N-terminal, the primer combination of CPCR-Nhis (forward) and CPCR-CXho (reverse) were used (see 2.3.3). The PCR reaction set-up for both the genes was the same, which is as follows:

PCR reaction set-up:

<u>Components</u>	<u>Volume</u>	<u>Concentration</u>
TripleMaster Polymerase Mix (5 U/μl)	0.5 μl	0.05 U/μl
Dra I digested library	5 μl	1.5 ng
Forward primer (5 pmole/μl)	2 μl	10 pmole
Reverse primer (5 pmole/μl)	2 μl	10 pmole
Sterile deionized H ₂ O	up to 50 μl	--
Total volume	50 μl	

The PCR cyclic parameters for amplification of CpSADH and CPCR with His-tags were:

<u>Steps</u>	<u>CpSADH-his</u>	<u>CPCR-his</u>	<u>Time</u>	<u>Cycles</u>
Denaturation	94 °C	94 °C	2 min	1
Denaturation	94 °C	94 °C	20 sec	32
Annealing	50 °C	55 °C	20 sec	
Elongation	72 °C	72 °C	1 min	
Final elongation	72 °C	72 °C	1 min	1
Cooling	4 °C	4 °C	∞	--

2.3.5 Agarose gel electrophoresis

Analytical as well as preparative gel electrophoresis of double-stranded DNA fragments were performed in 0.5-1.5% agarose gels (Aaij and Borst, 1972; Helling et al., 1974; Sambrook et al., 1989) supplemented with ethidium bromide (final concentration 0.5 μg/ml). The agarose was dissolved in 1x TAE buffer. Before loading on the gel, the DNA samples were mixed with 1 x DNA-loading buffer (end concentration). For determination of fragment size and concentration estimation, a defined amount of DNA size marker (GeneRuler™ 1 kb DNA Ladder) was included. Bands were visualized using a UV transilluminator at 312 nm. In preparative electrophoresis, the desired DNA fragment was excised using a scalpel under the

UV. The excised fragment isolated from the gel was then purified with the “E.N.Z.A. Gel-extraction kit” (PEQLAB).

50 x TAE buffer, pH 8 (1 liter)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA	18.6 g
Deionized water up to 1 l	

6 x DNA-loading buffer

Tris-HCl (pH 7.6)	10 mM
Glycerol	60% (v/v)
EDTA	60 mM
Bromophenol blue	0.03% (w/v)

2.3.6 Plasmid DNA isolation from *E.coli*

Alkaline lysis:

For screening of positive transformants, alkaline lysis (Birnboim and Doly, 1979) was done for plasmid isolation. The method involves 3 steps: first washing with RNase solution, second lysis of the cells with lysis buffer and third precipitation with Na-acetate. A single colony was inoculated in 5 ml of LB_(antibiotic) medium and grown overnight at 37 °C. The overnight culture was harvested by centrifugation for 10 min at 5000 rpm. The supernatant was discarded and the pellets were suspended by vortexing in 400 µl of ice-cold Solution A. To the suspension, 800 µl of Solution B was added and mixed by inverting the tube several times. Care should be taken not to vortex the suspension. The mixture was incubated on ice for 5 min and then, 600 µl of ice-cold Solution C was added and mixed by inverting the tube several times. A second incubation was done on ice for 15 min followed by centrifugation (4 °C, 13000 rpm, 10 min). 400 µl of the supernatant was taken in a fresh microcentrifuge and 260 µl of 2-propanol was added and incubated at –20 °C for 2 h. The DNA was collected by centrifugation (4 °C, 13000 rpm, 15 min). The supernatant was discarded carefully and the DNA was washed with 100 µl of 70% ethanol and centrifuged in vacuum till all of the ethanol was removed. The isolated plasmid DNA was then suspended in 50-100 µl of 10 mM Tris-HCl (pH 8). With the help of restriction analysis (2.3.7) and agarose gel electrophoresis (2.3.5), the plasmid-DNA was checked for insertion of the vector/insert constructs.

Solution A:

Glucose	50 mM
EDTA	10 mM
Tris-HCl (pH 8)	25 mM
RNase A/ml	100 µg

Solution B:

NaOH	0.2 N
SDS	1% (w/v)

Solution C:

Sodium acetate (pH 4.8)	3 M
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Plasmid MiniPrep Kit:

Different amounts of plasmid DNA after the alkaline lysis could be isolated through the membrane columns (Ish-Horowitz and Burke, 1981) of E.N.Z.A. Plasmid Miniprep Kit I (PEQLAB). 5 ml of the overnight culture of *E.coli* was centrifuged (10 min, 5000 rpm, 4 °C) and the plasmid-DNA was isolated following the manufacturer's instructions. The eluted DNA was quite concentrated and clean for further cloning, restriction analysis, transformation or sequencing.

2.3.7 DNA restriction digestion

Digestion of the DNA with restriction endonucleases was performed in the buffer supplied with the restriction enzyme and in accordance with the suppliers' recommendations for temperatures and duration of digestion. All the restriction endonucleases were bought from MBI Fermentas. Mostly digestion was done for 4-5 h (in case of EcoRI, PstI and HindIII) or up to 16 h (in case of NdeI and XhoI) using 10-20 U (EcoRI, PstI and HindIII) or 5-6 U (NdeI and XhoI) of the enzyme and 0.5-1.5 µg DNA. The digestion reaction was incubated at 37 °C and after completion of the restriction digestion, the reaction mix was analyzed by agarose gel electrophoresis (2.3.5). For preparative restriction digestion e.g., cloning of DNA fragments, reaction mix was purified with the PCR Purification kit (Qiagen) and quantified by agarose gel electrophoresis.

2.3.8 5' Dephosphorylation of DNA fragments

The digested and linearized plasmids were dephosphorylated at its 5'-termini directly after restriction digestion with Calf Intestine Alkaline Phosphatase (CIAP, MBI Fermentas) for 60-90 min at 37 °C with 1 U of CIAP in accordance with the suppliers' recommendations. All the dephosphorylated linearized plasmid DNA was purified with the PCR Purification kit (Qiagen) directly after the dephosphorylation was completed.

2.3.9 Ligation of DNA fragments**PCR Cloning Plus Kit (Qiagen):**

This kit was used for UA cloning. For that, the DNA fragment (PCR product) was ligated into the pDrive cloning vector supplied with the kit. The DNA ligation reaction was carried out in a total volume of 10 µl. The protocol was followed as mentioned in the user's manual. The

insert to vector molar ratio was adjusted to 8:1. The formula applied for estimating the concentration (in ng) of the insert/PCR product to be used for the ligation is as follows:

$$\text{ng of the vector/PCR product required} = \frac{\text{Conc. of the vector (ng)} \times \text{PCR product size (bp)} \times \text{molar ratio}}{\text{Size of the vector (bp)}}$$

Ligation of DNA fragments (“sticky-ends”):

For other successive ligation reactions of the insert to vectors (e.g. pKK223-3 or pET26b+), a 20 µl reaction volume was used with 5:1 molar ratio of insert to vector, 2-3 U of T4 DNA Ligase and 1x Ligation buffer (MBI Fermentas). The ligation mixture was incubated at 16-22 °C overnight (16-18 h). Thermal inactivation was done at 65 °C for 20 min. A control ligation was always done with the vector alone without any insert to check for self-ligation of the vector. After ligation reaction was completed, the mix was used for transformation (2.3.10).

2.3.10 Transformation and selection

Blue-white selection:

The blue-white selection is a method of differentiating transformants that carry the vector-insert construct to those that do not carry any insert by using X-Gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside) and IPTG (Isopropyl-[beta]-D-thiogalactopyranoside) as selection markers (Ullmann et al., 1967). The pDrive cloning vector (Qiagen) is capable of blue-white selection and was used for selection of positive clones. Transformation of the plasmid was done using QIAGEN EZ competent cells (ready-to-use) according to the manufacturer's instructions. As blue white selection was used, therefore X-Gal (80 µg/ml LB agar) and IPTG (0.05 mM final concentration) were included in the LB agar plates along with ampicillin (100 µg/ml LB agar). The plates were incubated at room temperature until the transformation mixture had absorbed into the agar. After that, the plates were inverted and incubated at 37 °C overnight (16-18 h). For blue-white selection, a second incubation at 4 °C was done for a few hours. This cold incubation enhances blue color development and thereby facilitates differentiation between blue colonies and white colonies.

Transformation with heat-shock:

Method 1: About 10 µl of the ligation mix or 10-20 ng of the circular vector DNA was added to 190 µl of thawed competent cells on ice and mixed by gentle tapping of the microcentrifuge tubes. This mix was incubated on ice for 30 min and then a heat shock was

given at 42 °C for 90 sec, after which a second incubation was done on ice for 5 min. To this, 800 µl of LB medium (containing 20 mM MgSO₄ and 10 mM KCl) was added and regeneration was done at 37 °C, 1200 rpm for 2 h on a thermo-mixer (Eppendorf). The transformation mixture was plated onto LB agar plates containing appropriate antibiotic for selection and incubated at 37 °C overnight (Hanahan, 1983).

Method 2: Alternatively, about 2-6 µl of the ligation mix was added to a prechilled 15-ml polypropylene tube and placed on ice. The competent cells were thawed on ice and mixed gently by mild tapping of the tubes. 100-200 µl of the cells were then transferred to the prepared tubes containing 2-6 µl of the ligation mix. This was gently mixed and incubated on ice for 20 min. A heat shock was given to the cells thereafter for 65 sec on a prewarmed water bath set exactly at 42 °C (without shaking), after which the tube was immediately placed back on ice for 2 min. To this, 900 µl room temperature SOC medium (2.1.3) was added and regeneration was done on a shaker incubator at 37 °C and 200-250 rpm for 1.5-2 h.

2.3.11 Preparation of DNase free RNase

The commercially available RNase was made DNase free by incubating the RNase stock solution at 95 °C for 15 min.

2.3.12 Sequencing

For determination of the nucleotide sequences of DNA fragments of vector/insert constructs, sequencing was done by Sequiserve (Vaterstetten, Germany). Before sequencing, the recombinant plasmid DNA was purified with E.N.Z.A. Plasmid Miniprep kit (PEQLAB).

2.3.13 Description of cloning set-up of recombinant CPCR and CpSADH

Expression vectors	Cloning/Expression Strains (<i>E.coli</i>)	Primers (Forward, Reverse)	Restriction sites, His ₆ -tag position
pKK223-3	JM109	CPCR-F1A CPCR-F2	EcoRI PstI
pET-26b(+)	DH5 α , BL21(DE3)	CPCR-Nhis CPCR-CXho	NdeI, N-terminal XhoI

Table 1: Cloning set-up of recombinant CPCR (excluding genome walking).

Expression vectors	Cloning/Expression Strains (<i>E.coli</i>)	Primers (Forward, Reverse)	Restriction sites, His ₆ -tag position
pKK223-3	JM109	ADH-J1 ADH-J2	EcoRI HindIII
pET-26b(+)	DH5 α , BL21(DE3)	JADH-NHis JADH-CXho	NdeI, N-terminal XhoI

Table 2: Cloning set-up of recombinant CpSADH.

2.4 Biochemical and analytical methods

2.4.1 Determination of protein

Bradford method (Bradford, 1976) was used to measure the protein concentration of solutions with the help of spectrophotometer or microplate reader. This is based on the coomassie blue dye-binding assay in which a differential color change of the dye occurs in response to various concentrations of protein. The maximum absorbance of the dye shifts from 465 nm to 595 nm when binding of protein occurs. The complex between the coloring dye and protein is formed after about 2 minutes and is stable for one hour in solution. The increase of the extinction is measured at 595 nm.

For determination of protein, 200 μ l of Bio-Rad reagent was mixed with 800 μ l of protein solution. Since the absorption is proportional to the protein quantity, the concentration of the protein solution can be determined over a linear calibration curve. The calibration curve was obtained with known protein concentrations of standard protein namely, bovine serum albumin (BSA, albumin fraction V) by reading the absorbance of the diluted BSA at 595 nm. The absorbance versus protein concentration curve was linear in the restricted protein concentration range (between 1 mg and 20 mg protein/ml sample solution).

Alternatively for the determination of smaller protein concentrations, a microtiterplate assay was accomplished. For this, the Bio-Rad coloring dye was diluted in the ratio of 1:5 with deionized water. For determination of the calibration curve, standard protein solutions (BSA) was used within a concentration range between 0.05 and 0.5 mg/ml. 200 μ l of the diluted dye was mixed with 10 μ l of protein solutions and after 10 min to 20 min, the extinction was determined at 595 nm. Using the calibration curve, the protein concentration of the unknown protein solution was determined. All the measurements were done in triplicate and the average value was calculated and used.

2.4.2 Determination of enzyme activity

Activity test for reduction:

The reducing activity of enzymes (CPCR/CpSADH) in aqueous medium was determined spectrophotometrically by measuring the rate of consumption of the cofactor $\text{NADH} + \text{H}^+$ at 340 nm. Acetyl-butyric-acid-ethyl ester (ABEE) was used as a standard substrate, which is reduced through the oxidation of the cofactor. The measurement was carried out for 1 min at 37 °C and pH 7. 1 Unit (U) of enzyme is defined as the amount of enzyme required to transform 1 μ mol of ABEE per minute under the experimental conditions. The substrate spectrum of the enzyme was determined with this method.

Standard enzyme test: 880 μ l buffer (100 mM TEA-HCl, pH 7)
 100 μ l ABEE solution (80 mM in 100 mM TEA-HCl, pH 7)
 10 μ l $\text{NADH} + \text{H}^+$ (20 mM in 100 mM TEA-HCl, pH 7)
 10 μ l CPCR/CpSADH solution

The volumetric activity of the enzyme was determined by equation (1)

$$\text{Volumetric activity} = \frac{V_{\text{total}} \cdot dA/dt \cdot df}{V_{\text{pip}} \cdot \epsilon \cdot d} \quad (\text{U/ml}) \quad (1)$$

V_{total}	Total volume of reaction (μl)
dA/dt	Difference in the extinction per min (min^{-1})
V_{pip}	Volume of enzyme solution (μl)
ϵ	Extinction coefficient of NADH at 340 nm ($6.22 \text{ l mmol}^{-1} \text{ cm}^{-1}$)
d	Path length of cuvette (1cm) In case of microtiterplate the path length is 0.26 cm for 100 μl volume and 0.67 cm for 200 μl reaction volume
df	Dilution factor

The specific activity of enzyme was determined by equation (2)

$$\text{Specific activity} = \frac{\text{Volumetric activity}}{\text{Protein concentration}} \quad (\text{U/mg}) \quad (2)$$

In order to determine the activity of large number of samples, e.g. during measurement of enzyme characteristics, activity tests in microtiterplate was carried out. The method was optimized for the specific enzymes.

Reaction for microtiterplate: 80 μl ABEE solution (100 mM in 100 mM TEA-HCl, pH 7)
10 μl NADH + H^+ (12.5 mM in 100 mM TEA-HCl, pH 7)
10 μl CPCR/CpSADH solution

Activity test for oxidation:

For determination of oxidizing activity of the enzyme, the method was used as described by Kojima (Kojima et al., 1995).

Standard reaction mixture: 50 mM Tris-HCl (pH 9)
50 mM (*S*)-1,3-butanediol
2.5 mM NAD^+
10 μl enzyme solution

The reaction was carried out in a total volume of 1 ml in cuvette at 30 °C for 1 min. The rate of NAD^+ reduction was followed at 340 nm.

Colorimetric activity test for CpSADH:

The method for the colorimetric activity test was used as developed by Steinsiek (Steinsiek, 2006). The test was accomplished with native protein gels. The activity for NAD^+ reduction was made visible in the protein bands. With the oxidation of (*S*)-1-phenylethanol, reduced

NADH transfers the electrons to phenazinmethosulphate, which converts the coloring dye iodonitrotetrazoliumviolet to insoluble, red formazane (Blaich, 1987; Peters, 1993). The band, in which the protein with the appropriate activity is located, colors itself dark red.

Assay for coloring activity: 100 mM TEA-HCl, pH 7
30 mM (*S*)-1-phenylethanol
0.1 mM NAD⁺
0.2 mg/ml phenazinmethosulphate
0.5 mg/ml iodonitrotetrazoliumviolet

After the native gel electrophoresis, the gels were incubated in the above reaction solution in darkness for 5-10 min.

2.4.3 Protein purification

Anion exchange chromatography:

In ionic exchange chromatography, the protein is bound to the matrix through electrostatic interaction (Rossomando, 1990). The material used here for the chromatographic purification (MacroPrep High Q) is a strong anion exchanger made of copolymer beads of methacrylate with quaternary ammonium ions as functional group. The adsorbed molecules could be eluted with the change in the column conditions, by mixing two buffers with different salt concentrations over step-gradient through continuous (linear) gradient with a FPLC system. The recombinant CpSADH (JM109pKK223-3CpSADH18 construct) was purified by this method. The first eluted fraction of protein with weak ions was achieved at 100 mM NaCl concentration. Thereby the NaCl concentration was increased in steps of 200 mM, 300 mM and 400 mM. The rest of the cell proteins were washed and eluted out of the matrix by increasing the NaCl concentration to 1 M. Fractions were collected at the rate of 12 ml with the help of auto-sampler and analyzed with SDS-PAGE (2.4.4) and also with the standard activity assays (2.4.2) and protein assays (2.4.1). The eluted fractions between 200-300 mM of NaCl concentrations showed activity for CpSADH. The flow rate of all the different steps was kept at 2 ml/min except for the loading step. The different steps for the purification were:

Equilibration: Before loading the protein, the column with the matrix was always equilibrated with the Loading/Binding buffer A for 5 column volumes
Loading: 1 ml/min for 2 loop-volumes
Binding/washing: 2 ml/min for 3 column volumes with Buffer A

Elution:	2 ml/min for 1.5 column volumes for every gradient step with different concentrations of NaCl in the Elution buffer B (100-400 mM)
Final washing:	2 ml/min for 5 column volumes with the Elution buffer B (up to 1 M NaCl concentration)
Equilibration:	2 ml/min for 3 column volumes with Buffer A

<u>Loading/Binding buffer A</u>	50 mM TEA-HCl (pH 8.3)
---------------------------------	------------------------

<u>Elution buffer B</u>	50 mM TEA-HCl (pH 8.3)
	1 M NaCl

The buffer compositions were optimized for binding of the specific protein (recombinant CpSADH).

Immobilized metal affinity chromatography (IMAC) with Ni-NTA:

Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins in 1975 (Porath et al., 1975) using the chelating ligand iminodiacetic acid (IDA). IDA was charged with metal ions such as Zn^{2+} , Cu^{2+} , or Ni^{2+} , and then used to purify a variety of different proteins and peptides (Sulkowski, 1985). Compared to the 3 metal-chelating sites of IDA that cannot tightly bind metal ions, Nitrilotriacetic acid (NTA, Qiagen) which is a tetradentate chelating adsorbent occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the His₆-tags. NTA binds metal ions far more stable than other available chelating resins (Hochuli, 1989) and retains the ions under a wide variety of conditions, especially under stringent wash conditions. NTA matrices can therefore bind His₆-tagged proteins very tightly and allow purification of proteins very easily through elution with an increasing concentration of imidazole. Ni-NTA superflow and Ni-NTA agarose (Qiagen) were used for the purification of the N-terminal His₆-tagged recombinant CpSADH [BL21(DE3)pET26b+CpSADH28his construct] under native conditions. Ni-NTA agarose was used for batch-purification of the protein in small scales. For preparative purification, Ni-NTA superflow was used and the protein was purified with the FPLC system in one step. The background proteins that were non-specific and bound to the matrix with a low-affinity were first eluted at 10 mM imidazole concentration. CpSADH was eluted in a very pure form by increasing the imidazole concentration to 250 mM. The flow rate and the different steps of purification were:

Equilibration:	2 ml/min for 5 column volumes with Buffer A (100%)
Loading:	1 ml/min for 2 loop volumes
Binding/Washing:	1 ml/min for 5 column volumes with Buffer A (100%) until A_{280} is stable
Elution:	2 ml/min for 5 column volumes with Buffer B (100%). Protein is eluted within the first 1-3 column volumes. The next 2 column volumes is for final washing
Equilibration:	2 ml/min for 2-3 column volumes with Buffer A (100%)

Buffer A (Lysis/Binding/Washing) 1 liter:

50 mM NaH_2PO_4	6.90 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
10 mM imidazole	0.68 g imidazole (MW 68.08 g/mol)

The pH was adjusted to 8.0 using NaOH.

Buffer B (Elution buffer) 1 liter:

50 mM NaH_2PO_4	6.90 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
250 mM imidazole	17.00 g imidazole (MW 68.08 g/mol)

The pH was adjusted to 8.0 using NaOH.

Ultrafiltration:

For buffer exchange, desalting and concentration of the eluted protein fractions containing enzymatic activity from anion exchange and IMAC, the protein fractions were pooled and concentrated by centrifugation (5000 rpm, 4 °C) with the Vivaspin 20 ml concentrators (Vivascience) having a molecular weight cut-off (MWCO) of 10000 Daltons. The protein was concentrated to the desired level, the filtrate was removed and the concentrator was refilled with the desired buffer (100 mM TEA-HCl, pH 7.5). The sample was then again concentrated and the process was repeated until the salt concentration was sufficiently removed. Typically, 3 wash cycles were carried out. The final concentrated and desalted recombinant CpSADH dissolved in 100 mM TEA-HCl, pH 7.5, was either stored at -20 °C in 50% glycerol (v/v) or lyophilized by freeze-drying and the enzyme powder was then stored at -20 °C.

2.4.4 Gel electrophoresis

SDS-PAGE:

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the separation of proteins by discontinued polyacrylamide gel (Laemmli, 1970). For this, 12% polyacrylamide gels were made. The 12% resolving gel was over-laid with 2-propanol to ensure a flat surface and to exclude air. After polymerization 2-propanol was removed, the sample combs were attached and 5% stacking (loading) gel was poured. The protein samples for analysis were mixed in 1:1 ratio with the reduced sample buffer Roti[®]Load1 (4x) (Roth) and subjected to heat denaturation at 95 °C for 5-7 min. The samples were then cooled and centrifuged shortly. 10-20 µl of each of the prepared samples were pipetted in every comb. As a molecular weight standard, 5 µl of the protein marker PageRuler[™] Prestained Protein Ladder (MBI Fermentas) was also loaded. The separation of the protein was carried out at a constant voltage supply of either 150 or 180 V for 60-90 min. For visualization of the protein bands, the gels were first washed with deionized water and then stained with PageBlue[™] Protein Staining Solution (MBI Fermentas) for 60 min to overnight. Since polymerization takes place immediately after the addition of APS and TEMED, these components were directly added just before pouring the gels after a quick mixing.

Composition of the resolving and stacking gels for SDS/Native-PAGE were as follows:

Components	Resolving gel [ml]		Stacking gel (5%) [ml]
	(12%)	(7.5%)	
40% Acrylamide mix	3.0	1.9	0.5
1.5 M Tris-HCl, pH 8.8	2.5	2.5	—
1 M Tris-HCl, pH 6.8	—	—	0.5
10% (w/v) SDS	0.1	—	0.04
10% (w/v) APS	0.1	0.1	0.04
TEMED	0.004	0.004	0.004
Deionized H ₂ O	4.3	5.5	2.9
Total volume	10	10	4

SDS running buffer:

Tris	3 g/l
Glycine	14.4 g/l
SDS	1 g/l

Native-PAGE:

In native PAGE, the protein is not denatured and remains in its active form (Schägger and Jagow, 1991). Therefore the gel contains no SDS and the charge of the proteins in the gel depends on their isoelectric point and the pH of the used buffer. Corresponding to SDS-gels, 5% stacking gel and 7.5% resolving gel was made as described earlier. SDS was substituted with deionized water both in the gels and in the running buffer. The protein samples were mixed in the ratio of 1:1 with the native sample buffer and directly loaded on the gel slots. The separation was done for 4-5 h at a constant voltage of 100 V. In order to prevent activity loss through over-heating, the gel chamber was placed on an ice-bath. The gels were then directly used for activity staining (2.4.2; colorimetric activity test for CpSADH).

2.4.5 Transfer of protein (Western Blot)

The principle of immunoblotting method (Kyhse-Andersen, 1984) lies in the discontinued transfer of protein on membranes. The protein bands of the SDS-polyacrylamide gel were transferred by western blot to a PVDF membrane (Invitrogen) by running for 80 min with a constant voltage of 30 V. The protein-free areas of the transfer membrane were saturated by incubation for 1 hour in a blocking solution composed of 3% BSA in TBS buffer. The membrane was thereafter treated with a solution containing antibody-conjugate from anti-His₆-peroxidase for 1 hour. The membrane was then washed three times for 5 min in TBS-Tween and finally soaked in 1 x DAB solution. After 1-10 min (depending on the intensity of the staining), the enzymatic reaction was stopped by repeated washing with deionized water.

Transfer buffer:

5 x SDS running buffer	20% (v/v)
Methanol	10% (v/v)

TBS buffer:

Tris-HCl, pH 7.2	10 mM
NaCl	150 mM

TBS-Tween:

Tris-HCl, pH 7.2	10 mM
NaCl	150 mM
Tween 20	0.1% (v/v)

DAB solution:

DAB substrate (10 x)	10%
Peroxide buffer	90%

Antibody solution:

Anti-His ₆ -antibody conjugate	40 µl
BSA	1%

2.4.6 Chromatographic analysis of substrate and product

HPLC:

The conversion of acetophenone into phenylethanol by CpSADH in aqueous system was analyzed with HPLC using a Nucleosil 100/5C18 column (250 x 4 mm) and a UV detector 166 (Beckmann Coulter). The running solvents used were 60% H₂SO₄ at pH 3 and 40% acetonitrile with a flow rate of 0.6 ml/min and a column temperature of 40 °C. The injection volume and wavelength used were 20 µl and 220 nm respectively.

GC:

The conversion of substrate to product in organic solvent (hexane) was analyzed with GC using decane as internal standard. For this, the FS-FFAP-CB-0.25 column was used which has a length of 25 m and an internal diameter of 0.25 mm. The detector used was FID (Flame ionization detector). The injector and detector temperatures were set at 220 °C. A split of 1:50 and a pressure of 60 kPa with nitrogen as carrier gas were used. The temperature program was used as developed and used by Steinsiek (2006).

Original oven temperature:	40 °C for 4 minutes
Temperature increase:	20 °C per minute till 100 °C 30 °C per minute till 180 °C
End temperature:	180 °C for 7 minutes
Total time duration:	16.7 minutes

The retention times of hexane was 1.2-1.5 min, acetone was 1.6 min, 2-propanol was 2.2 min, decane was 2.95 min, acetophenone was 10.1 min and phenylethanol was 11.0 min.

Stereoselective synthesis by CpSADH was analyzed by a chiral GC column to determine the enantiomeric purity of the product. For this, FS-Cyclodex-beta-I/P capillary column was used with a split of 1:30 and a pressure of 100 kPa with nitrogen as carrier gas. The temperature program was: 100 °C for 3 min; 30 °C per minute till 150 °C; 150 °C for 4 min. Under these conditions, retention of acetophenone was 7 min and (*S*)-1-phenylethanol was 8.1 min.

2.4.7 Inhibition study

The inhibition of CpSADH when exposed to different reagents was studied by the method as described by Kojima (Kojima et al., 1995). For this, a definite amount of the enzyme was

incubated at 30 °C for 30 min with different reagents and the residual activity was measured using the activity assay for microtiterplate (2.4.2) in a volume of 100 µl. As a control, the same amount of enzyme (untreated) was incubated without any reagent for the same time and temperature. The untreated enzyme was then compared to the treated ones.

2.4.8 Temperature optimum and activation energy

In order to determine the temperature optimum of recombinant CpSADH with and without His₆-tag, the activity of the enzyme was measured spectrophotometrically for 1 min by following the oxidation of NADH at 340 nm in different temperatures ranging from 25-58 °C; using the standard activity assay conditions for microtiterplate (2.4.2). All the solutions were heated to the above temperatures before adding the enzyme. The activation energy was calculated based on the Arrhenius equation (equation 3) that is,

$$k = A \cdot e^{-E_A/RT} \quad (3)$$

k	=	Rate constant
A	=	Pre-exponential factor
E_A	=	Activation Energy (J mol ⁻¹)
R	=	Gas constant (8.314 J mol ⁻¹ K ⁻¹)
T	=	Absolute temperature (K ⁻¹)

From the temperature-dependent rate constants of the linear range of the optimum curve, the activation energy can be calculated by plotting $\ln k$ against the reciprocal value of the absolute temperature. The gradient of the straight line determined from the diagram equals to $-E_A \cdot R^{-1}$.

2.4.9 Temperature stability and half-life

The temperature stability of recombinant CpSADH was determined by incubating the enzyme solution (2 U/ml) in 100 mM TEA-HCl (pH 7.5) for a period of 24 h at a temperature ranging from 25-45 °C and measuring the residual activity at regular time intervals spectrophotometrically at 340 nm for 2 min and 37 °C by following the oxidation of NADH using the standard activity assay for microtiterplate (2.4.2). The half-life of the enzyme was calculated over the inactivation constant that is given in equation (4). By plotting the natural logarithm of the activity against the time the inactivation constant can be determined as the slope.

$$A = A_0 \cdot e^{-kt} \quad (4)$$

A	=	Activity at time t
A_0	=	Activity at the beginning of measurement at t_0
k	=	Inactivation constant
t	=	Time

The half-life τ can be calculate by the following equation (5)

$$\tau = \frac{\ln 2}{k} \quad (5)$$

2.4.10 pH optimum and pH stability

The optimum pH of the recombinant CpSADH was determined spectrophotometrically. Substrate, cofactor (NADH) and enzyme were dissolved in 100 mM TEA-buffer with different pH ranging from 5.5-10 and the activity was measured for 2 min at 37 °C using the standard activity test for microtiterplate (2.4.2) by following the oxidation of NADH at 340 nm.

The pH stability was determined by incubating the enzyme (2 U/ml) in 100 mM TEA-buffer with different pH ranging from 5.5-8.5 for a period of 24 h at 25 °C. The residual activity was measured at regular time intervals at 37 °C for 2 min and 340 nm spectrophotometrically using the standard activity test for microtiterplate.

2.4.11 Stability in hexane

The half-life of the recombinant CpSADH with His₆-tag in direct contact with the organic solvent was analyzed in an aqueous-organic two-phase system. The aqueous phase consisted of a definite amount of lyophilized CpSADH powder (2 U) after IMAC purification in 100 mM TEA-HCl, pH 7.5 and the organic phase consisted of hexane. The hexane phase consisted of a volume of 25, 50 and 75% in a total volume of 1 ml. The aqueous phase was mixed with a magnetic stirrer and the hexane phase was lain over it. The reaction vials were stirred at 25 °C without inter-mixing the two phases. In regular time intervals, each of the reaction vials was withdrawn, the lower (aqueous) phase was taken off and the residual activity was determined photometrically.

The stability of the enzyme was also determined in hexane alone. For this, lyophilized CpSADH powder (2 U) was incubated in this organic solvent in reaction vials and after regular time intervals each of the reaction vials was withdrawn, the enzyme was extracted with 100 mM TEA-HCl, pH 7.5 and the residual activity was measured photometrically and compared to the untreated enzyme powder.

2.4.12 Synthesis in aqueous system

In order to determine the conversion of acetophenone in purely aqueous system, enzymatic synthesis was carried out at 30 °C in a reaction volume of 1 ml consisting of 0.5 U of recombinant CpSADH, 10% (v/v) 2-propanol for cofactor regeneration and 1 mM NADH as cofactor. Furthermore, to determine the influence of 2-propanol on the activity of the enzyme, 2-propanol was used in a concentration of 5 to 20% (v/v). As the solubility of acetophenone in buffer is only about 45 mM, therefore this concentration was used in the synthetic reactions. After the reaction was completed, the enzyme was inactivated by heating at 95 °C for 5 min, and the concentration of substrate and product was measured via HPLC (2.4.6).

2.4.13 Synthesis in liquid two-phase system

The activity of the CpSADH in a purely organic reaction medium was examined with the enzyme lyophilized together with NADH based on the method described by Steinsiek (2006). For that 1 U of IMAC purified CpSADH together with 10 µmole of NADH were lyophilized directly in the reaction vials and the reaction was started by adding 1 ml of the organic solvent, consisting of hexane with 200 mM acetophenone and 10 mM decane. Two sets were examined without additional water, two with 0.1% (v/v) water, two with 0.5% (v/v) water and another two with 1% (v/v) water. To one set in each case, 1% (v/v) of 2-propanol was added. The reaction vials were mixed on an overhead shaker and after 48 h the product formation was determined by GC.

Biocatalytic synthesis in aqueous-organic two-phase system using the recombinant CpSADH with His₆-tag was carried out according to the procedure described by Steinsiek (Steinsiek, 2006). For this, different percentages of hexane as organic phase were used (2.4.11) in a total reaction volume of 1 ml. The aqueous phase consisted of enzyme, 1 mM NADH dissolved in 100 mM TEA-HCl (pH 7.5). The enzyme concentration used was 0.5 U per ml and was based on the total reaction volume.

The organic phase consisted of 10 mM decane (calculated over the organic phase) as internal standard, acetophenone as substrate. The concentration of the substrate was calculated over the aqueous and organic phases. For cofactor regeneration, 2-propanol was used in a concentration of 10% (v/v) of the aqueous phase. The concentrations of substrate and product in aqueous phase were measured by HPLC, and in organic phase by GC (2.4.6).

2.4.14 Substrate spectrum of recombinant CpSADH

The conversion of different substrates was determined photometrically at 340 nm over the oxidation of the cofactor $\text{NADH} + \text{H}^+$. The substrates were dissolved in a concentration of 10 mM in 100 mM TEA-HCl, pH 7 and the relative activity in comparison to the standard substrate ABEE was analyzed. The activity assay consisted of 8 mM of each substrate (from 10 mM stock solution), 0.2 mM NADH and 10 μl of enzyme solution in a total volume of 1 ml and the activity was measured at 37 °C for 1 min.

2.5 Immobilization of recombinant CpSADH

2.5.1 Immobilization in polyvinyl alcohol (PVA)

The immobilization of the enzyme together with the cofactor in the PVA matrix was carried out by the method as described by Steinsiek (Steinsiek, 2006). For this, first a 12% (w/v) polyvinyl alcohol (PVA) solution was prepared and afterwards diluted with the enzyme solution to 9%. 3 g PVA MW 10-98 together with 3 g polyethylene glycol (PEG 1000) and 21 ml of 100 mM TEA-buffer pH 7.5 were heated up to 90 °C and the solution was dissolved with continuous stirring. After the cooling of the solution to room temperature, 2 ml of 1.5 M NaOH were dropped carefully into the solution under vigorous stirring. This was done to ensure complete saponification of the polyvinyl alcohol (Prüsse, 2000). After about 30 minutes of stirring, the pH value was adjusted to pH 7.5 with concentrated hydrochloric acid solution. For the production of beads, the polyvinyl alcohol solution (after saponification and pH adjustment) was mixed with the enzyme solution in a ratio of 3:1. For the standard reaction, 2 units of enzyme solution in 100 mM TEA-HCl pH 7.5 with 1 mM $\text{NADH} + \text{H}^+$ per ml of beads were used. Beads of PVA-enzyme solution were produced via introduction of the solution in drops into prechilled (-70 °C) silicone oil and following a slow thawing process (overnight at 4 °C). Before using the beads for synthesis, they were slowly warmed up to room temperature and washed with hexane.

The activity of immobilized enzymes cannot be determined directly, since the substrate and/or product concentrations in the hydrogel cannot be detected. By determining the balanced distribution, the final concentrations of substrate and product that are distributed in the organic phase and hydrogel phase, can be calculated. This equilibrium position was quantified with enzyme-free immobilisates over the reduction of the concentrations in the organic phase, after the equilibrium was reached. For this, 1 gram immobilisate in the ratio of 1:3 in hexane with varying substrate and/or product concentrations were used. The size of the beads was about 10 μ l regulated over the dropping volume.

Standard reaction conditions for PVA beads: Unless otherwise stated all syntheses with immobilized enzyme in PVA beads were carried out according to the following assay conditions: 1 gram immobilisate, 10 mM decane, 3.3% 2-propanol (v/v), 200 mM acetophenone as substrate.

Hexane was used as the organic phase. Decane was used as an internal standard in a concentration of 10 mM added to the organic phase. The immobilisate was used in a ratio of 1:3 to the organic phase. The concentration of 2-propanol that was added into the organic phase is related to the volume of the aqueous phase (hydrogel phase), such that its concentration in the aqueous phase amounts to 10%. Enzymatic synthesis was carried out at 25 °C by stirring in an over-head shaker for proper mixing of the two phases.

2.5.2 Immobilization in Sepabeads

SEPABEADS[®] EC-SERIES (Resindion S.r.l., Italy) are ready-to-use enzyme carriers. Two types of the Sepabeads series namely, EC-HFA and EC-EP were used to immobilize the enzyme. SEPABEADS[®] EC-HFA are crosslinked copolymer of methacrylate containing amino and epoxy groups whereas SEPABEADS[®] EC-EP are crosslinked copolymer of methacrylate containing oxirane (epoxy) groups.

Immobilization procedure:

For SEPABEADS[®] EC-EP, 10 units of the enzyme (IMAC purified) dissolved in 4 ml of 200 mM potassium phosphate buffer (pH 8) were added to 1 gram of sepabeads. In case of SEPABEADS[®] EC-HFA, the same units of enzyme were dissolved in 4 ml of 20 mM potassium phosphate buffer (pH 7.6). The buffer compositions were optimized for each of the two types of beads. The ratio of enzyme solution to beads was always kept at 4:1. The enzyme

solution was stirred in an overhead shaker at 25 °C for about 2 hours (till the adsorption was completed). During this time, the remaining activity was checked in the solution every 1 hour. After the adsorption was completed, the immobilization (for covalent binding) was proceeded at 5 °C for 48 h. The supernatant was eliminated after checking the residual activity and the immobilized enzyme was washed thoroughly five times with deionized water and five times with 50 mM potassium phosphate buffer (pH 7.6).

Standard reaction conditions for Sepabeads: A ratio of 0.0625 wet biocatalyst to the total reaction volume was used. This ratio was calculated by dividing the amount of wet biocatalyst (in gram) with the total reaction volume (in ml). Unless otherwise stated all synthetic reactions with the enzyme immobilized Sepabeads were carried out as follows:

1. Reaction set-up for aqueous system

250 mg beads

1 mM NADH

10% 2-propanol (v/v)

45 mM acetophenone in 50 mM potassium phosphate buffer, pH 7.6

The reaction was carried out in a total volume of 4 ml at 25 °C with proper mixing in an end-to-end shaker.

2. Reaction set-up for aqueous-organic system

In the immobilization method with Sepabeads, it was not possible to co-immobilize NADH like in the PVA beads. The NADH should be freely available to reach the active center of the enzyme and was therefore supplied in a small aqueous phase. Two types of reaction conditions were set up as described:

SET A:

250 mg beads

Aqueous phase (1 ml) consisting of,

1 mM NADH dissolved in 50 mM potassium phosphate buffer, pH 7.6

Organic phase (3 ml) consisting of,

3.33% 2-propanol (v/v)

10 mM decane

200 mM acetophenone as substrate

SET B:

250 mg beads

Aqueous phase (0.5 ml) consisting of,

1 mM NADH dissolved in 50 mM potassium phosphate buffer, pH 7.6

Organic phase (3.5 ml) consisting of,

1.42% 2-propanol (v/v)

10 mM decane

200 mM acetophenone as substrate

The reactions were carried out in a total volume of 4 ml at 25 °C.

Hexane was used as the organic phase. Decane was used as an internal standard in a concentration of 10 mM (calculated over the organic phase) added to the organic phase. The concentration of 2-propanol that was added into the organic phase is related to the volume of the aqueous phase, such that its concentration in the aqueous phase amounts to 10%. The substrate concentration was calculated over the aqueous and organic phases and a final concentration of 200 mM was used. To ensure proper mixing of the two phases, the reaction vials were stirred in an end-to-end shaker.

It was found that the substrate and/or product when used showed some affinity towards the Sepabeads. Some amounts of the substrate and/or product were always bound to the carriers. In order to quantify the material balance, the same amount (250 mg) of enzyme-free beads were used in a reaction volume of 4 ml containing varying concentrations of substrate and product. After the equilibrium was reached, the final concentrations of substrate/product present in the reaction volume were measured with HPLC (aqueous phase) and with GC (organic phase).

2.5.3 Stability of the immobilized CpSADH in Sepabeads

The stability of the CpSADH immobilized in the Sepabeads was determined over the half-life of the immobilized enzyme in an aqueous-organic two-phase system. For this, 250 mg of the immobilisate were stored in an aqueous-organic phase in a ratio of 1:3 (1 ml buffer: 3 ml hexane). The reaction vials were stirred in an end-to-end shaker and were incubated at 25 °C, 30 °C and 37 °C. In regular time intervals (after every 24 h), each of the reaction vials (from each temperature) was taken out and the aqueous-hexane phases were carefully thrown away. The remaining amounts of the aqueous-organic phases were sucked away with filter papers. The reaction was thereafter started by freshly adding another 1 ml buffer consisting of 1 mM NADH, and 3 ml hexane consisting of 266 mM acetophenone as substrate and 10 mM decane as internal standard. The final NADH concentration was therefore 1 mM (calculated over the aqueous phase), that of decane was 10 mM (calculated over the organic phase) and that of acetophenone was 200 mM (calculated over the aqueous-organic phase). The reaction was accomplished at 25 °C with stirring and after 24 hours the substrate and product concentrations were determined.

3 Results and Discussion

3.1 Cloning of CPCR and CpSADH

3.1.1 Isolation of genomic DNA from *C.parapsilosis*

Based on the method as described in section (2.3.1) the genomic DNA (gDNA) from *C.parapsilosis* DSMZ 70125 strain was isolated with a very high purity. The isolated gDNA had a size of about 23 kb (23000 bp) and a concentration of 400 ng/μl and was also intact without any smears. The isolated gDNA was used as the template for the construction of libraries with the GenomeWalker kit. The following figure shows the gel document of the isolated gDNA. The genomic DNA of *C.parapsilosis* isolated by Bönitz (Bönitz, 2001) was 7500 bp only, which is about 15.5 kb less as compared to the DNA that has been isolated in this work. The reason could be shearing of DNA during the isolation process.

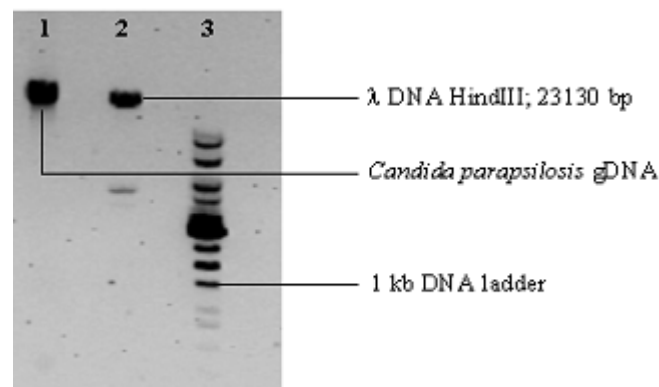


Figure 4: Genomic DNA of *C.parapsilosis*. Lane 1= *C.parapsilosis* gDNA; Lane 2 = λ DNA Hind III marker; Lane 3 = GeneRuler™ 1 kb DNA ladder. 1 μl of the isolated genomic DNA was loaded in 0.5% agarose/EtBr gel.

3.1.2 Construction of libraries

Four different libraries were constructed from the total genomic DNA of *C.parapsilosis* by digesting the gDNA with EcoRV, DraI, StuI and PvuII (Blunt-end cutters). Of the four libraries, the one digested with StuI was only partially digested, whereas all the other three were fully digested. It seemed that there may be no or only few restriction sites for StuI in the gDNA of *C.parapsilosis*. The positive control library (consisting of human gDNA) was constructed by digesting with PvuII.

The following Figure 5 illustrates the different libraries produced.

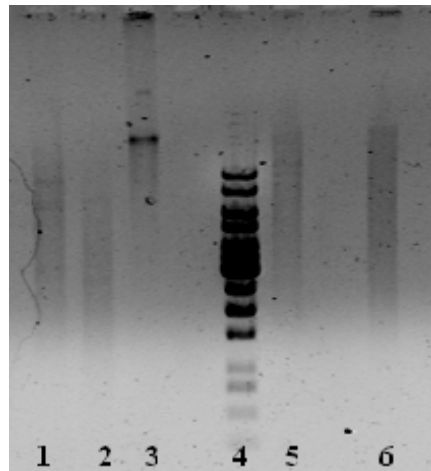


Figure 5: Gel documentation of the digested genomic DNA from *C.parapsilosis*. Lane 1 = library digested with EcoRV; Lane 2 = library digested with DraI; Lane 3 = library digested with StuI; Lane 4 = GeneRuler™ 1 kb DNA ladder; Lane 5 = library digested with PvuII; Lane 6 = control human genomic DNA library digested with PvuII. 5 µl of the digested genomic DNA libraries were loaded in 0.5% agarose/EtBr gel.

The above figure shows that the library digested with StuI (Lane 3) is only partially digested as compared to other libraries. The digestion time with StuI was prolonged in order to see if the enzyme digests the DNA fully, but it also resulted in partial digestion.

The libraries were purified with phenol: chloroform extraction and were then ligated to the GenomeWalker adaptors according to the supplier's instructions. These adaptor-ligated libraries were used for genome walking with PCR.

3.1.3 Analysis of the partial amino acids sequences of wtCPCR

For genome walking of the *C.parapsilosis* gDNA to amplify the CPCR gene, the partial protein sequences of the wtCPCR (Bönitz, 2001) were analyzed in order to determine the homologies with other ADHs and also to predict probable primer regions for genome walking. The partial amino acids sequences and the derived nucleotide sequences of the wtCPCR from the N-terminal towards the C-terminal are described in the next page. The source of information of the gene sequences was created by considering the codon usage of *C.parapsilosis* and degeneration of the genetic code.

CPCR-Sequence 1(N-terminal)

PEIPKTQKAVVFETSGGKLEYKDIPVPKPKSNELLINVKYSGVCHTDLHAW

Gene sequence 1(N-terminal)

5'-CCA-GAA-ATT-CCA-AAA-ACT-CAA-AAA-GCT-GTT-GTT-TTT-GAA-ACT-TCA-GGT-GGT-AAA-TTG-GAA-TAC-AAA-GAT-ATT-CCA-GTT-CCA-AAA-CCA-AAA-TCA-AAT-CAA-TTG-TTG-ATT-AAT-GTT-AAA-TAC-TCA-GGT-GTT-TGT-CAC-ACT-GAT-TTG-CAC-GCT-TGG-3'

CPCR-Sequence 2

AAPILCAGVTYK

Gene sequence 2

5'-GCT-GCT-CCA-ATT-TTG-TGT-GCT-GGT-GTT-ACT-GTT-TAC-AAA-3'

CPCR-Sequence 3

AGEWVCISGAGGGLGSLAIQYAIA

Gene sequence 3

5'-GCT-GGT-GAA-TGG-GTT-TGT-ATT-TCA-GGT-GCT-GGT-GGT-GGT-TTG-GGT-TCA-TTG-GCT-ATT-CAA-TAC-GCT-ATT-GCT-3'

CPCR-Sequence 4

SLGAEAYIDFTKEKDIVEAV

Gene sequence 4

5'-TCA-TTG-GGT-GCT-GAA-GCT-TAC-ATT-GAT-TTT-ACT-AAA-GAA-AAA-GAT-ATT-GTT-GAA-GCT-GTT-3'

CPCR-Sequence 5

VVAPVFDVVKSV EIKGSYVGNRKDTQEALDFFARGKVK

Gene sequence 5

5'-GTT-GTT-GCT-CCA-GTT-TTT-GAT-GTT-GTT-AAA-TCA-GTT-GAA-ATT-AAA-GGT-TCA-TAC-GTT-GGT-AAT-AGA-AAA-GAT-ACT-CAA-GAA-GCT-TTG-GAT-TTT-TTT-GCT-AGA-GGT-AAA-GTT-AAA-3'

CPCR-Sequence 6 (C-terminal)

IVGLSELPEVFKLMEEGKILGRYVLDTSK

Gene sequence 6(C-terminal)

5'-ATT-GTT-GGT-TTG-TCA-GAA-TTG-CCA-GAA-GTT-TTT-AAA-TTG-ATG-GAA-GGT-AAA-ATT-TTG-GGT-AGA-TAC-GTT-TTG-GAT-ACT-TCA-AAA-3'

Each of the above 6 sequences were aligned using the databases (ExPASy, NCBI). It was found that all of them had homologies in different percentage with other alcohol dehydrogenases of *Candida albicans*, *Saccharomyces cerevisiae*, *Pichia stipitis* (yeast), the highest being with the ADH1 of *C.albicans* (85.5%).

The individual gene sequences were then compared with a patented ADH of *C.parapsilosis* (Kojima et al., 1995). It was found that in Gene sequence 1, 20 bases in between the fragments 59-78 has 77% homology with the said enzyme in between 531-510. In Gene sequence 2, 28 bases in between the fragments 12-39 has 74% homology with the said enzyme in between 577-606. In Gene sequence 3, 28 bases in between the fragments 18-45 has 75% homology with the said enzyme in between 528-549. In Gene sequence 4, 20 bases in between the fragments 8-27 has 70% homology with the said enzyme in between 718-735. In Gene sequence 5, 23 bases in between the fragments 13-35 has 76% homology with the said enzyme in between 701-719. In Gene sequence 6, 21 bases in between the fragments 66-86 has 77% homology with the said enzyme in between 750-730. This homology study showed that the partial amino acids sequences of the wtCPCR had some homologies in some parts of the sequences with this patented ADH, though in general both the sequences were quite different from each other.

The probable primer regions for genome walking were determined taking the gene sequences (derived from the partial amino acids sequences) of the wtCPCR into consideration. Gene sequence 3 was selected for designing the primers. This sequence was chosen because of low homologies to other ADHs to rule out unspecific binding. Moreover the N-terminal sequence derived by Bönitz (2001) was not intended to be used in this work, because this had already been done by Bönitz with doubtful success. Furthermore, it was not really expected that the same sequence would be found but rather with an extension at the N-terminal. As this sequence was somewhere in the middle of the wtCPCR gene (since it was neither in the N- nor towards the C-terminal), the primers were designed as such that the walking of the gene

with the Genome Walker kit would take place upstream i.e. towards the N-terminal in order to amplify the start (5'-end) of the CPCPCR gene.

3.1.4 PCR amplification of CPCPCR from the libraries and subcloning in pDrive vector

In her work Bönitz (2001) cloned a DNA sequence coding for 349 amino acids, which was believed to be the complete CPCPCR gene. But, from the substrate spectrum analysis it was found that the enzyme considered as CPCPCR was not so. The probable reason could be that the sequence cloned by Bönitz was not the complete sequence. It was also not clear if it was the right gene though the DNA sequence had high homologies with other ADHs. In this work, a method was needed so as to get the whole CPCPCR gene sequence. As such the genome walking technique was applied primarily because the N-terminal sequence derived by Bönitz was not intended to be used.

The four different libraries constructed were amplified using the primer combinations of SET A and of SET B (2.3.3) as gene specific primers and the adaptor primers that were supplied with the GenomeWalker kit. In the first instance, the PCR amplification of the libraries were carried out with the SET A primers such that, the genome walking would take place upstream in order to identify the start and open reading frame of the CPCPCR gene (5'-end) and followed by walking downstream to amplify the end (3'-end) with the SET B primers. Using the primer combination of SET A as gene specific primers and the PCR cycles as described earlier (see 2.3.4; PCR set-up for the amplification of the constructed libraries with the Genome Walker kit using adaptor primers), the libraries were walked upstream. The primary PCR consisted of the primer CPCPCR-R1b and adaptor primer 1 (AP1) [outer primers] while the nested PCR consisted of the primer CPCPCR-R2b and adaptor primer 2 (AP2) [inner primers]. In order to increase the specificity of the PCR products and to minimize unwanted amplifications, the products of the primary PCR were used as the template for the nested PCR. Figure 6 depicts the amplified PCR products of the nested PCR.

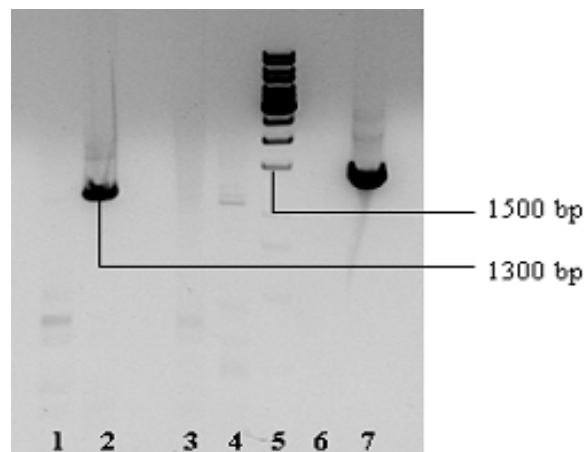


Figure 6: Gel documentation of the products of nested TD-PCR. Lane 1 = library digested with EcoRV; Lane 2 = library digested with DraI; Lane 3 = library digested with StuI; Lane 4 = library digested with PvuII; Lane 5 = GeneRuler™ 1 kb DNA Ladder; Lane 6 = negative control without template DNA; Lane 7 = positive control (control human genomic DNA library digested with PvuII). 5µl of the digested genomic DNA libraries were loaded in 1.5% agarose/EtBr gel.

As can be seen from Figure 6, of the four libraries, a major amplification of a DNA fragment of about 1300 bp was found to be amplified in the DraI digested library. A very small amplification of the same range (about 1300 bp) was also found to be amplified by the PvuII library. The rest of the libraries showed little or no amplification at all. The possible reason could be that the nucleotide sequence coding for CPCr must have restriction sites for EcoRV, StuI and PvuII within it. Therefore, digesting the gDNA with these enzymes resulted in the cleavage within the CPCr gene and thus fragmentation. The PCR product of 1300 bp amplified by the DraI library was subcloned into the pDrive vector (Qiagen) via UA-cloning in which the A-overhang of the PCR product hybridize to the U-overhang of the vector. The cloned product was sequenced at Sequiserve (Vaterstetten, Germany). The sequenced DNA fragment is shown in Figure 7.

```

1 GAATTGTGCG GCCATTTAGG TGACACTATA GAATACAGCG GCCGCGAGCT CGGGCCCCCA
61 CACGTGTGGT CTAGAGCTAG CCTAGGCTCG AGAAGCTTGT CGACGAATTC AGATTACTAT
121 AGGGCACGCG TGGTCGACGG CCCGGGCTGG TAAATCATGC GATAAACTC GGAGTAACAA
181 AGTCCGCAAT CTGGTTTTTT TTATAATCAC ACACAAAAAA CACTGAGGTA GAACAAAATC
241 CACAAACCCA CAAACACACA AACCACAAA AGAACAAAAG AACAAATGTAA CCCACCCAGA
301 TCCAGTCACC CACTCACACA CCTACAACTT TCATCAGATC ATTTCCGAAT TGGRAAAAAA
361 AGTCATCCTA TACAAAAATG TACACGCTCA CACAAAACCTC ACTTCTTTAT TACTATTCCA
421 TATAGTATTA GAAACGGAGT GGGTGAATGT ATTCTTGCA GATAAGCGCT TCAACATTAT
481 GCATGAACCA AAAACACCAA CATGTATCAG AATAACATTC ACCCGCAAAA AAAAGCATAA
541 AATCATGCAT ATCCGATGGT TGAATAATTT TTATTTTCAA AAAGCYAAAT GTAACAATAT
601 AAATAGACCA CGAGGGAATG CCAATTGGCT CAACACATTT CTTTCATTTT AAAAGCTTTA
661 TTAGATCAAT TGCAGTTAAC AAAGTAACTA CTATTCCAAT TAAAGCGTTC ACTACTACTA
721 CAACTAAAAC TAAAACTAAA ATTACAACCTA CAACTACAAC AAACAGTTTT ATATCTACAA
781 TGCCAGAAAT TCCAAAGACC CAAAAAGCTG TTGTGTTTGA AACAAGCGGA GGTAAATTAG
841 AATACAAGGA TATTCCAGTC CCCAAACCAA AATCAAATGA ATTGTTGATT AATGTCAAGT
901 ACTCTGGTGT GTGCCATACT GATTTACACG CTTGGAAGAG TGATTGGCCA TTGGACACCA
961 AATTACCCCT TGTGGTGGT CATGAGGGTG CTGGTGTGTG TGTGGCATG GGTGATAATG
1021 TCAAAGGATG GGAATTTGGT GATTATGCTG GTATCAAATG GTTGAATGGC TCTTGTTTGA
1081 ATTGTTGAATT TTGTGAACAA GGAGCTGAAC CAAATTGCCC TCAAGCTGAC TTGTCTGGTT
1141 ACACCCATGA TGGYTCAATC GAACAATATG CTACTGCTGA TGCTGTCCAA GCTGCTAGAA
1201 TCCCCAAGAA TGCTGATTTG GCTAAAGCTG CTCCAATTTT GTGTGCTGGT GTTACTGTAT
1261 ACAAGGCTTT GAAGACTGCT CAATTGAAGG CTGGTGAATG GGTTTGATTT TCWGGTGCTG
1321 GTGGTGGTTT GAATCACGAA TTCTGGATCC GATACGTAAC CCGTCTGCAG CATGCGTGGT
1381 ACCGAGCTT

```

Figure 7: Nucleotide sequence of the first DNA fragment. The highlighted grey region represents the ORF starting with an ATG.

The ORF (Open reading frame) of the sequence began with an ATG that codes for methionine. The ORF was read from 5'-3' Frame 3 (start-618; end-1388). The translated amino acid sequence beginning from position 618 is as follows:

CPCR- HALF:

*MPIGSTHFFHFKSFIRSLAVNKVTTIPIKAFTTTTTTKTKTKITTTTTNSFISTMPEIPKTQKAV
VFETSGGKLEYKDIPVPKPKSNELLINVKYSGVCHTDLHAWKGDWPLDTKLPLVGGHEGA
GVVVGMDNVKGWEIGDYAGIKWLNGSCLNCFCEQGAEPNCPQADLSGYTHDXSFEQ
YATADAVQAARIPKNADLAKAAPILCAGVTYKALKTAQLKAGEWVCIXGAGGGLNHEFWI
RYVTRLQHAWYRA (257 amino acids)*

Global alignment of this amino acid sequence resulted in close matches of approximately 40% with ADH2 (Taylor et al., 1998 in GenBank; Jones et al., 2004) and ADH1 (Bertram et al., 1996; Shen et al., 1991) of *C.albicans* and the ADH of *P.stipitis* (Passoth et al., 1998).

Since the walking of CPCR was done upstream to get the start of the gene, it was presumed that the above amino acid sequence (CPCR-HALF) is nearly the half of the whole CPCR gene.

Another genome walking (downstream) was done to amplify the 3'-end of the CPCR gene, using the primer combinations of CPCR-V1 and adaptor primer 1 (AP1) for primary PCR; and CPCR-V2 and adaptor primer 2 (AP2) for nested PCR. The PCR cycles used are described in the section (2.3.4; PCR set-up for the amplification of the constructed libraries with the Genome Walker kit using adaptor primers). The first gene specific primer, CPCR-V1 was constructed slightly upstream of the start codon, ATG, in between the base region 591-620 (Figure 7). The nested gene specific primer, CPCR-V2 was constructed starting with the start codon, ATG, in between the base region 618-647 (Figure 7). The *Dra*I digested library was used as the template because the previous amplified product was obtained from it as well. A 2000 bp DNA fragment was amplified which was subcloned again into the pDrive vector. The gel documentation of the amplified PCR product and also the sequenced DNA fragment thereof are described in the following figures (Figure 8 and Figure 9).

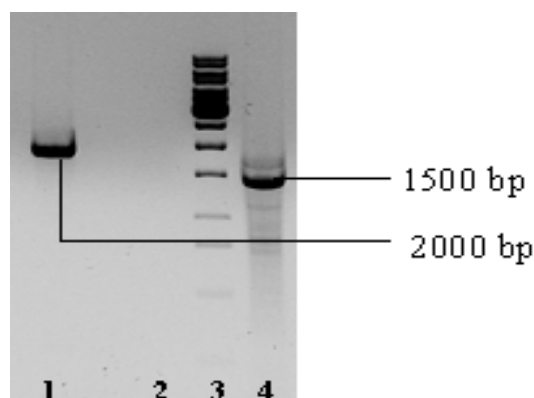


Figure 8: Gel documentation of the nested TD-PCR product. Lane 1 = amplified product from the *Dra*I digested library; Lane 2 = negative control; Lane 3 = GeneRuler™ 1 kb DNA Ladder; Lane 4 = positive control.

The DNA sequence of the amplified product is as follows:

```

1 GAATTGTGCG GCCATTTAGG TGACACTATA GAATACAGCG GCCGCGAGCT CGGGCCCCCA
61 CACGTGTGGT CTAGAGCTAG CCTAGGCTCG AGAAGCTTGT CGACGAATTC AGATTATGCC
121 AATTGGCTCA ACACATTTCT TTCATTTCAA AAGCTTTATT AGATCAATTG CAGTTAACAA
181 AGTAACTACT ATTCCAATTA AAGCGTTCAC TACTACTACA ACTAAAAC TAACATAAAAT
241 TACAAC TACAACAA ACAGTTTAT ATCTACAATG CCAGAAATTC CAAAGACCCA
301 AAAAGCTGTT GTGTTTGA CAAGCGGAGG TAAATTAGAA TACAAGGATA TTCCAGTCCC
361 CAAACCAAAA TCAAAATGAAT TGTTGATTAA TGTCAGTAC TCTGGTGTGT GCCACTACTGA
421 TTTACACGCT TGGAAAGGTG ATTGGCCATT GGACACCAAA TTACCCCTTG TTGGTGGTCA
481 TGAGGGTGCT GGTGTTGTTG TTGGCATGGG TGATAATGTC AAAGGATGGG AAATTGGTGA
541 TTATGCTGGT ATCAAATGGT TGAATGGCTC TTGTTTGAAT TGTGAATTTT GTGAACAAGG
601 AGCTGAACCA AATTGCCCTC AAGCTGACTT GTCTGGTTAC ACCCATGATG GTTCATTTCGA
661 ACAATATGCT ACTGCTGATG CTGTCCAAGC TGCTAGAATC CCCAAGAATG CTGATTTGGC
721 TAAAGCTGCT CCAATTTTGT GTGCTGGTGT TACTGTATAC AAGGCTTTGA AGACTGCTCA
781 ATTGAAGGCT GGTGAATGGG TTTGTATTTC TGGTGTGGT GGTGGATTGG GTTCATTGGC
841 TATTCAATAC GCTATTGCCA TGGGTTACAG AGTTATTGGT ATCGATGGAG GTGCTGAAAA
901 GGGTGAATAC ATTAAATCTT TAGGAGCTGA AGCTTATATT GACTTTACCA AGGAAAAAGA
961 TATTGTCGAA GCTGTTAAAA AGGCAACTAA TGGTGGCCCA CATGGAGTTA TCAATGTGTC
1021 TGTTTCCGAA AAGGCCATCA ATCAATCAGT TGAATATGTT AGACCATTGG GTAAAGTTGT
1081 TCTTGTGGT TTACCAGCTG GATCCAAAGT TGTTGCCCT GTTTTCGATG CTGTTGTTAA
1141 ATCAGTTGAA ATTAAAGGTT CTTATGTTGG TAACAGAAAG GATACTCAAG AGGCTTGGGA
1201 TTTCTTTGCT AGAGGTAAAG TCAATTGTCA AATCAAGATT GTTGGTCTTA GTGAATTACC
1261 AGAAGTTTTC AAATTGATGG AAGAAGGTAA GATTTTGGGA AGATACGTAC TTGACACCAG
1321 CAAGTAAAAG AGTTAGAGAG TAGATCAAAT GTTTTGGTTT AATAGTTCTT CTTTATATT
1381 GACGAAATGA GATGGAATTT GGAAATGTG TAAAAAAGAG AAAAGAAATG TAGATTCAAC
1441 TTATACACAC TTTACTTGCA AGCTTGCAA AGCTGGAGGT TATTTTCCTA ATCAACGCTT
1501 ATCTTTTCA ATTCTATCAC GCAACACATT CAATTGTATC TGATTGTGCA AGTTTTCATC
1561 ATTAATCAAT TCAATAGTTT TGTTCTTTTC CTTCAATTCA ATACTCAAGT GTTCAATTTG
1621 CTTTGTGAAT TTACTAACCA ATTTTTCCAA TTTGCCAATT CGTACATCCT TATCTTTAAT
1681 TATAAACTCT AGTTTCTCAT TTTTCAAAGT AATTGAGTTC AAATTTGTAA TCAAGTCTTC
1741 ATTTTCATTT CGTAGTTGAT GATTTTCCTT AATGATTACA ACTGTGGCGG CATCATTATC
1801 ACTAATGTTG TCCTTTAGTT TATCTTGTG AACACTCTTT GACTTTGATT TAGTGCTGGG
1861 CGGGACTTTT TCTTTTTCGA TTCCTAAGT TGTCGATGGA GATGGTGACT TGGAGCTAAA
1921 TGGAGTTTCG TCCTCCTCCT CTGTGTTACT TTGGCAATGA TGATGATGAT GTTGCTGTTT
1981 TTTCAGAAAG AGCTTTTCCA CTAATTGTTT ACCAGCCCGG GCCGTCGACC ACGCGTGCCC
2041 TATAGTAATC ACGAATCTG GATCCGATAC GTAACGCGTC TGCAGCATGC GTGGTACCGA
2101 GCTTT

```

Figure 9: Nucleotide sequence of the second DNA fragment. The highlighted grey region represents the full PCR gene with the start codon ATG and stop codon TAA.

The ORF with an ATG as start codon began from the region 116 and ended with a stop codon TAA (ochre) at position 1327 (Figure 9). Therefore the PCR began with an ATG that codes for methionine and ended with the stop codon TAA and constituted of 1212 bp. Further analysis of the DNA sequences upstream of the start codon ATG at position 618 (Figure 7), showed that some parts of the sequence had close homologies to that of different eukaryotic promoter regions (Altschul, et al. 1997), which meant that there were no additional DNA sequences upstream of this start codon that could be a part of this gene coding for PCR, and that the sequence that has been derived through genome walking is a complete sequence of PCR with a start and a stop codon.

CPCR-FULL:

Figure 10: Translated amino acid sequence of recombinant CPCR.

```
CPCR      VSVSEKAINQSVEYVRPLGKVVLVGLPAGSKVVPVFDVAVKSVSEIKGSYVGNRKDTQEA
ADH-2     VSVSERAIGQSTFEYVRTLGKVVLVGLPAGAKISTPFVDAVIKTIQIKGSYVGNRKDTAEA
ADH-1     VSVSEKAIDQSVEYVRPLGKVVLVGLPAHAKVTAPVFDVAVKSIEIKGSYVGNRKDTAEA
*****  * * * * * * * * * * * * * * * * * * * * * * * * * * *
```

```

CPCR      L D F F A R G K V N C Q I K I V G L S E L P E V F K L M E E G K I L G R Y V L D T S K
ADH-2     V D F F T R G L I K C P I K I V G L S E L P E V Y K L M E E G K I L G R Y V L D N D K
ADH-1     I D F F S R G L I K C P I K I V G L S D L P E V F K L M E E G K I L G R Y V L D T S K
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

```

Figure 11: Global alignment score of the CPCR with ADH1 and ADH2 from *Candida albicans*. The “*” represents close homologies with CPCR.

The CPCR therefore began with a methionine (M) followed by a proline (P) in the N-terminal. The total number of amino acids is 403. The C-terminal amino acid sequence of CPCR ended with a serine, lysine and followed by the stop codon TAA. Contrary to the start codon methionine, Bönitz (2001) reported that the N-terminal protein sequence of wtCPCR began with a proline instead, and therefore an amino acid triplet codon coding for methionine had to be added to the DNA sequence for the expression of the CPCR gene. This could signify that the N-terminal protein sequence of the wtCPCR was not complete.

In the work from Bönitz (Bönitz, 2001), the recombinant CPCR was described to have 349 amino acids which was derived from the same known partial protein sequences of the wtCPCR as has been described in section (3.1.3). But, using the same amino acid sequences as primers and with genome walking, a protein sequence of 403 amino acids was amplified which possessed close identities with the ADH1 and ADH2. The new derived CPCR in this work had an additional 54 amino acids “MPIGSTHFFHFKSFIRSIAVNKVTTIPIKAFTTTTTTKTKTKITTTTTTNSFIST” on its N-terminal. But the new recombinant CPCR protein also included all the known partial amino acids sequences of the wtCPCR and the complete CPCR protein sequence cloned by Bönitz. The following alignment (Figure 12) summarizes a comparison of the different known partial amino acids sequences (Sequence 1, 2,3,4,5 and 6; from N- towards C-terminal) of the wtCPCR, the CPCR cloned by Bönitz and the CPCR that had been derived in this work.

```

CPCR      MPIGSTHFFHFKSFIRSIAVNKVTTIPIKAFTTTTTTKTKTKITTTTTTNSFISTMPEIPK
CPCR-Bönitz -----MPEIPK
SEQ1      -----PEIPK
SEQ2      -----
SEQ3      -----
SEQ4      -----
SEQ5      -----
SEQ6      -----

CPCR      TQKAVVFETSGGKLEYKDIPVPKPKSNELLINVKYSVCHTDLHAWKGDWPLDTKLPLVG
CPCR-Bönitz TQKAVVFETSGGKLEYKDIPVPKPKSNELLINVKYSVCHTDLHAWKGDWPLDTKLPLVG
SEQ1      TQKAVVFETSGGKLEYKDIPVPKPKSNELLINVKYSVCHTDLHAW-----
SEQ2      -----
SEQ3      -----
SEQ4      -----
SEQ5      -----
SEQ6      -----

```

CPCR	GHEGAGVVVGMDNVKGWEIGDYAGIKWLNGSCLNCEFCEQGAEPNCPQADLSGYTHDGS
CPCR-Bonitz	GHEGAGVVVGMDNVKGWEIGDYAGIKWLNGSCLNCEFCEQGAEPNCPQADLSGYTHDGS
SEQ1	-----
SEQ2	-----
SEQ3	-----
SEQ4	-----
SEQ5	-----
SEQ6	-----
CPCR	FEQYATADAVQAARIPKNADLAKAAPILCAGVTVYKALKTAQLKAGEWVCISGAGGGLGS
CPCR-Bonitz	FEQYATADAVQAARIPKNADLAKAAPILCAGVTVYKALKTAQLKAGEWVCISGAGGGLGS
SEQ1	-----
SEQ2	-----AAPILCAGVTVYK-----
SEQ3	-----AGEWVCISGAGGGLGS-----
SEQ4	-----
SEQ5	-----
SEQ6	-----
CPCR	LAIQYAIAMGYRVIGIDGGAEKGEYIKSLGAEAYIDFTKEKDIVEAVKKATNGGPHGVIN
CPCR-Bonitz	LAIQYAIAMGYRVIGIDGGAEKGEYIKSLGAEAYIDFTKEKDIVEAVKKATNGGPHGVIN
SEQ1	-----
SEQ2	-----
SEQ3	LAIQYAIA-----
SEQ4	-----SLGAEAYIDFTKEKDIVEAV-----
SEQ5	-----
SEQ6	-----
CPCR	VSVSEKAINQSVEYVRPLGKVVVLVGLPAGSKVVAPVFDVAVKSVEIKGSYVGNRKDTQEA
CPCR-Bonitz	VSVSEKAINQSVEYVRPLGKVVVLVGLPAGSKVVAPVFDVAVKSVEIKGSYVGNRKDTQEA
SEQ1	-----
SEQ2	-----
SEQ3	-----
SEQ4	-----
SEQ5	-----VVAPVFD-VVKSVEIKGSYVGNRKDTQEA
SEQ6	-----
CPCR	LDFFARGKVNCQIKIVGLSELPEVFKLMEEGKILGRYVLDTSK
CPCR-Bonitz	LDFFARGKVNCQIKIVGLSELPEVFKLMEEGKILGRYVLDTSK
SEQ1	-----
SEQ2	-----
SEQ3	-----
SEQ4	-----
SEQ5	LDFFARGKV-----
SEQ6	-----IVGLSELPEVFKLMEEGKILGRYVLDTSK

Figure 12: Alignment of recombinant CPCR translated protein to that of different known partial protein sequences of wtCPCR gene and the CPCR cloned by Bönitz.

A reason behind this lack of 54 amino acids in the cloned CPCR from Bönitz could be that, when purifying the wtCPCR for sequencing the protein, this amino acid sequence somehow got detached maybe due to instability of the N-terminal amino acids of the wtCPCR itself, and therefore the CPCR gene cloned by Bönitz was not a complete one. Based on the homologies with other alcohol dehydrogenases as described above, and analysis with the partial protein sequences of wtCPCR and the cloned CPCR from Bönitz, makes it however possible to state that the gene that has been cloned in this work is an ADH from *C.parapsilosis*.

The derived 403 amino acid sequence of CPCR was further analyzed for possible glycosylation motifs to check if the CPCR is a glycoprotein. Glycosylation belongs to the post-translational modifications, through which a protein must pass after translation in order to fold it in its correct native condition if necessary. Glycosylation occurs widely in eukaryotes but very rarely in prokaryotes. When expressing a eukaryotic protein such as the CPCR in a prokaryotic host such as *E.coli*, it must be assured that such a modification is not required or important for the eukaryotic protein expression, otherwise it would not have been feasible for the expression of recombinant CPCR as an active enzyme in *E.coli*. Bönitz already reported in her work that the test for sugar with the wtCPCR showed no signal for glycosylation and confirmed that the carbonyl reductase possessed no sugar residues. In this work therefore, the possibilities for glycosylation were based only on sequence analysis. The 403 amino acids of CPCR were searched for possible N-linked glycosylation sites with the help of protein databases such as **NetNGlyc 1.0** server that predicts N-glycosylation sites in human protein. N-glycosylation motifs of N-G-S at position 150, N-V-S at position 300 and N-Q-S at position 309 were found to be present in the amino acid sequence of CPCR (Figure 10). For N-linked glycosylation, a tripeptide sequence, known as glycosylation *sequon* consisting of Asn-X-Ser or Asn-X-Thr, where X could be any amino acid except proline (Gavel and Von Heijne 1990) is necessary but not sufficient for glycosylation (Pless et al., 1977). Proteins without signal peptides are unlikely to be exposed to the N-glycosylation machinery and thus are not glycosylated even though they contain potential motifs. The 54 additional amino acids sequence that is present in the N-terminal of the recombinant CPCR was therefore analyzed for signal peptides using the database **SignalP** server that predicts for signal peptides in eukaryotes. It was found that the 54 amino acids sequences of CPCR was not a signal peptide (Nielsen et al., 1997) and were parts of the mature protein. Both the experimental analysis done by Bönitz (2001) and the sequence analysis confirmed that the CPCR was not a glycoprotein and could be therefore expressed as an active enzyme in an *E.coli* expression system.

3.1.5 Cloning of the 1212 bp CPCR gene into expression vectors

For expression of the recombinant CPCR gene in *E.coli*, the gene was cloned into two expression vectors namely pKK223-3 and pET-26b(+) under the control of two promoters: *TAC* (*lac-trp* hybrid promoter) and *T_{7lac}* (*T7-lac* hybrid promoter).

The pKK223-3 vector has the following structural elements:

1. A regular *TAC*-promoter element that is induced with IPTG (Isopropyl- β -D-thiogalactoside) for the expression of the target gene.
2. The *LacZ* ribosome-binding site followed by an ATG start codon.
3. A “Multiple cloning site”, into which the insertion of a foreign DNA can be done.
4. A strong transcription terminator, for e.g. the *rrnB* at the end of the “Multiple cloning site”.
5. A “Replication origin” (*ori*) and the β -Lactamase gene. This gene enables the selection of the recombinant bacterial clone over the ampicillin resistance (Sutcliffe, 1979).

The pET-26b(+) vector has the following structural elements:

1. A *T_{7lac}*-promoter element (Studier et al., 1990; Dubendorff and Studier, 1991) that is induced with IPTG for the expression of the target gene.
2. The ribosome-binding site is followed by a unique *NdeI* site for cloning into the ATG start codon at the 5'-end of the insert coding sequence.
3. A “Multiple cloning site”, into which the insertion of a foreign DNA can be done.
4. A strong transcription terminator, for e.g. the *T7* at the end of the “Multiple cloning site”.
5. A “Replication origin” (*ori*) and the kanamycin-coding gene. This gene enables the selection of the recombinant bacterial clone over the kanamycin resistance.

The recombinant CPCR had been able to be cloned in the correct orientation into the expression vectors and corresponding restriction sites for cloning were introduced at both ends of the gene. Apart from the desired orientation of the gene, a suitable distance of the start codon methionine (ATG) from the promoter could be kept and thus the ribosome also could bind on the plasmid.

For cloning into the pKK223-3 vector, an EcoRI site was added into the 5'-end of the CPCR gene. On the 3'-end of the gene, a PstI restriction site, as well as a stop codon TAA has been introduced (primer combinations: CPCR-F1A and CPCR-F2).

In the case of the pET-26b(+) vector, an NdeI site was added into the 5'-end of the CPCR gene. This vector was used for expression of the CPCR with His₆-tag. Therefore a His₆-tag on the N-terminal of the CPCR gene was introduced by adding six histidine residues just after the NdeI site on the 5'-end. On the 3'-end of the gene, an XhoI restriction site was introduced just after the stop codon TAA (primer combinations: CPCR-Nhis and CPCR-CXho).

In Figure 13, the restriction map of the recombinant CPCR gene derived from genome walking (Figure 9) is described. With the help of this map it was evident, which restriction enzymes cannot generally be used for cloning of the CPCR, since they would cause fragmentation of the gene.

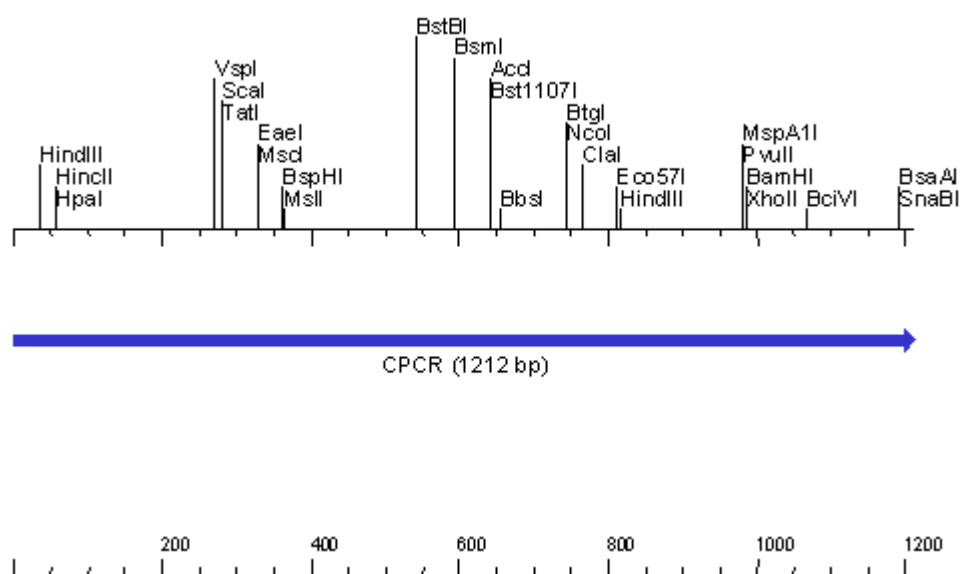


Figure 13: Map of the recombinant CPCR gene.

For cloning the CPCR gene into the expression vectors, apart from the insertion of the restriction sites, an error-free amplification of the PCR product was necessary. The occurrence of accidental mutations in the process of amplification, could lead to an inactive recombinant protein. The Taq-polymerase possesses no “proof-reading activity” (Flaman et al., 1994) and therefore the appearance of mutations during the process of amplification cannot be avoided. Due to this, the amplification of the PCR-products (CPCR as well as

CpSADH) for cloning in the expression vectors were carried out only with thermostable and proof-reading polymerases such as ISIS & GOTM Mastermix (Qbiogene) or TripleMaster[®] PCR System (Eppendorf). The DraI digested library was used as a template for PCR amplification of the CPCR gene.

The amplified CPCR fragment with the primer combinations of CPCR-F1A/CPCR-F2 (PCR cycles as described in section 2.3.4; PCR set-up for the amplification of CpSADH and CPCR without His₆-tag) and the pKK223-3 expression vector were after restriction digestion, ligated together and transformed into competent *E.coli* JM109 expression strain.

The amplified CPCR fragment with the primer combinations of CPCR-Nhis /CPCR-CXho (PCR cycles as described in section 2.3.4; PCR set-up for the amplification of CpSADH and CPCR with His₆-tag), and the pET-26b(+) expression vector were after restriction digestion, ligated together and transformed into competent *E.coli* BL21(DE3) expression strain .

In Figure 14(a), an agarose gel is represented, which shows the self-digested CPCR-PCR-product and the expression vector pKK223-3 that had been isolated in each case, after incubation with EcoRI and PstI. Whereas, in Figure 14(b), the represented agarose gel shows the self-digested CPCR-PCR-product and the expression vector pET-26b(+) that had been isolated, after incubation with NdeI and XhoI.

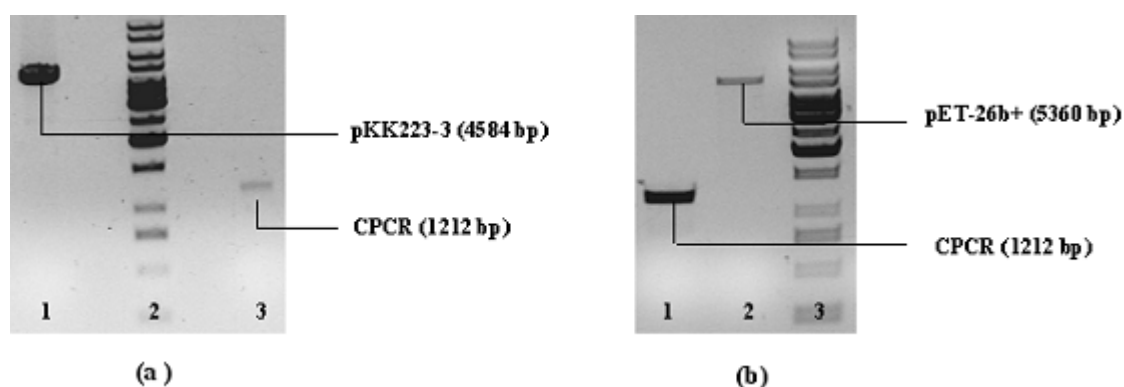


Figure 14

(a): Restriction digestion of the PCR amplified CPCR gene and the expression vector pKK223-3. Lane 1= Digested pKK223-3 with EcoRI and PstI for 4 h at 37 °C; Lane 2= GeneRulerTM 1 kb DNA ladder; Lane 3 = Digested CPCR fragment with EcoRI and PstI for 4 h at 37 °C.

(b): Restriction digestion of the PCR amplified CPCR gene and the expression vector pET-26b(+). Lane 1= Digested CPCR fragment with NdeI and XhoI for 16 h at 37 °C; Lane 2= Digested pET-26b(+) with NdeI and XhoI for 16 h at 37 °C; Lane 3 = GeneRulerTM 1 kb DNA ladder.

The plasmids that were isolated from the transformed bacteria are shown in Figure 15(a) for the recombinant vector construct pKK223-3CPCR9 and in Figure 15(b) for the recombinant vector construct pET26b+CPCR9his. The plasmids were likewise digested, as in this above example, as a check for successful ligation, with the restriction enzymes and isolated afterwards in agarose gels. Positive clones could be identified thereby as the plasmids with an increase in the size, which was due to the ligation of the CPCR gene into the plasmids (as shown in Figure 15 a and b). Before further use, positively tested clones in the restriction analysis, were finally confirmed through DNA sequencing for the completeness and correctness of the CPCR gene on molecular level and to exclude errors such as point mutations, etc.

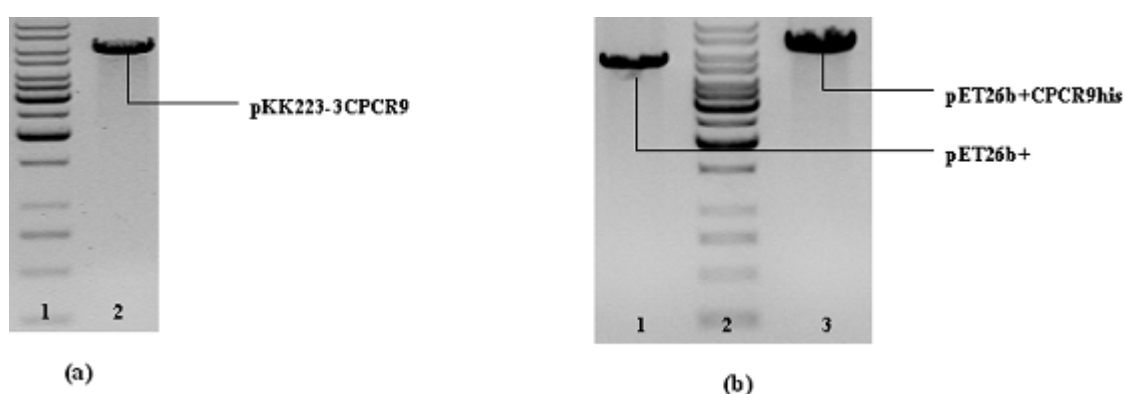


Figure 15

(a): Restriction digestion of the CPCR/pKK223-3 recombinant vector construct after successful ligation and cloning. Lane 1= GeneRulerTM 1 kb DNA ladder; Lane 2 = Digested pKK223-3CPCR9 construct with EcoRI for 4 h at 37 °C.

(b): Restriction digestion of the CPCR/pET-26b(+) recombinant vector construct after successful ligation and cloning. Lane 1= Digested pET-26b+ vector with NdeI for 4 h at 37 °C as an example of self-ligation possessing no insert within it; Lane 2= GeneRulerTM 1 kb DNA ladder; Lane 3 = Digested pET26b+CPCR9his construct with NdeI for 4 h at 37 °C.

3.1.6 Cloning of the 1011 bp CpSADH gene into expression vectors

The CpSADH gene coding for an alcohol dehydrogenase from *C.parapsilosis* IFO 1396 strain was first reported by Kojima et al. in the year 1995. The presence of this gene was searched for in the *C.parapsilosis* DSMZ 70125 strain. This was done by PCR amplification of the CpSADH gene using the DraI digested library as template and the primer combinations ADH-J1 and ADH-J2 (PCR cycles as described in section 2.3.4; PCR set-up for the amplification of

CpSADH and CPCR without His₆-tag). The amplified CpSADH fragment and the pKK223-3 expression vector were after restriction digestion, ligated together and transformed into competent *E.coli* JM109 expression strain.

Furthermore, the cloning of the said gene was also done by an addition of N-terminal six-histidine residues in order to facilitate easy purification of the recombinant protein. For this the said gene was amplified by PCR using the DraI digested library and the primer combinations of JADH-NHis and JADH-CXho (PCR cycles as described in section 2.3.4; PCR set-up for the amplification of CpSADH and CPCR with His₆-tag). The amplified fragment and the pET-26b(+) expression vector were after restriction digestion, ligated together and transformed into competent *E.coli* BL21(DE3) expression strain. At this point it should be stated that a powerful feature of the pET system is the ability to clone target genes under conditions of extremely low transcriptional activity, that is, in the absence of a source of T7 RNA polymerase. Background expression is minimal in the absence of T7 RNA polymerase because the host RNA polymerases do not initiate from T7 promoters and the cloning sites in pET plasmids are in regions weakly transcribed by read-through activity of bacterial RNA polymerase. Sometimes low levels of basal expression can cause difficulties in growth and plasmid instability in some expression hosts due to transcription from the T7 promoter in the pET plasmids. Therefore for initial cloning of the CpSADH (and also CPCR) into the pET-26b(+) and for maintaining the recombinant plasmids, the *E.coli* strain DH5 α was used because this bacterial strain is *recA*⁻ *endA*⁻ and has high transformation efficiencies and good plasmid yields. For expression of protein, the recombinant plasmids were transformed into the expression strain BL21(DE3).

For cloning into the pKK223-3 vector, an EcoRI site was added into the 5'-end of the CpSADH gene. On the 3'-end of the gene, a HindIII restriction site was added just after the stop codon TAG (primer combinations: ADH-J1 and ADH-J2).

In the case of the pET-26b(+) vector, an NdeI site was added into the 5'-end of the CpSADH gene. His₆-tag on the N-terminal of the CpSADH gene was introduced by adding six histidine residues just after the NdeI site on the 5'-end. On the 3'-end of the gene, an XhoI restriction site was introduced just after the stop codon TAG (primer combinations: JADH-NHis and JADH-CXho). The following restriction map of CpSADH was used to decide which restriction sites could be and which ones could not be used.

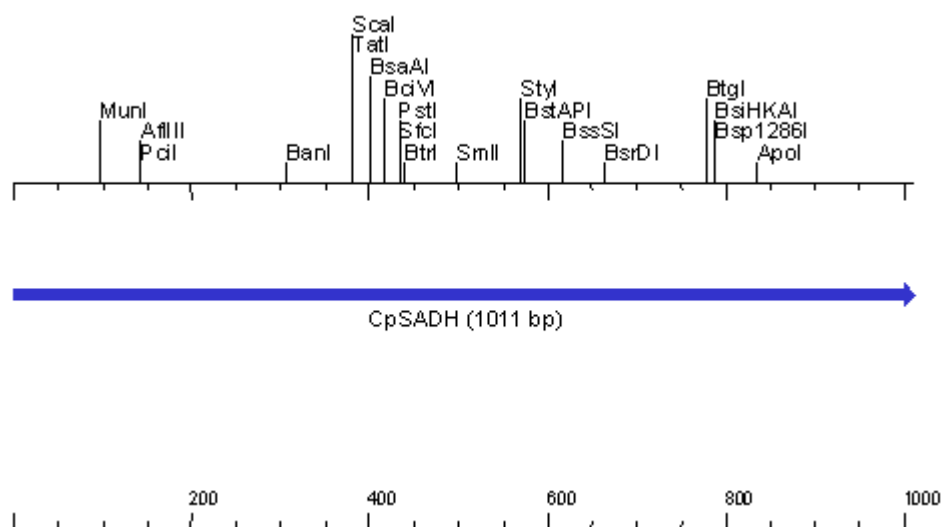
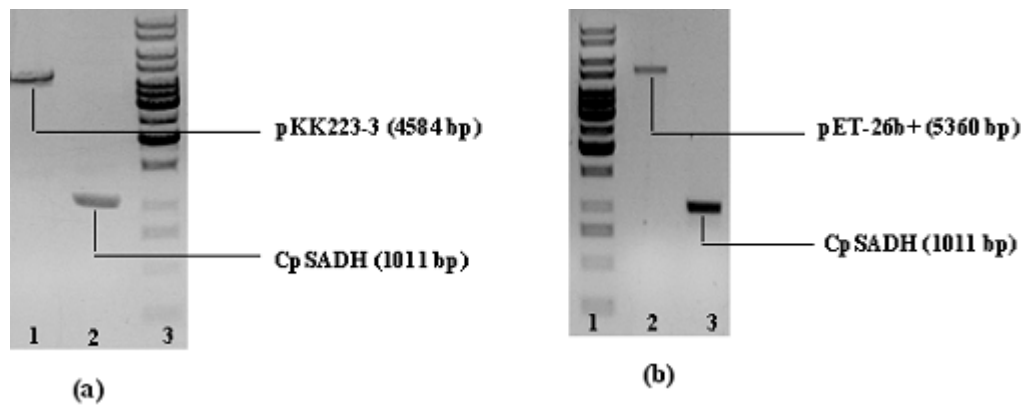


Figure 16: Restriction map of recombinant of CpSADH gene.

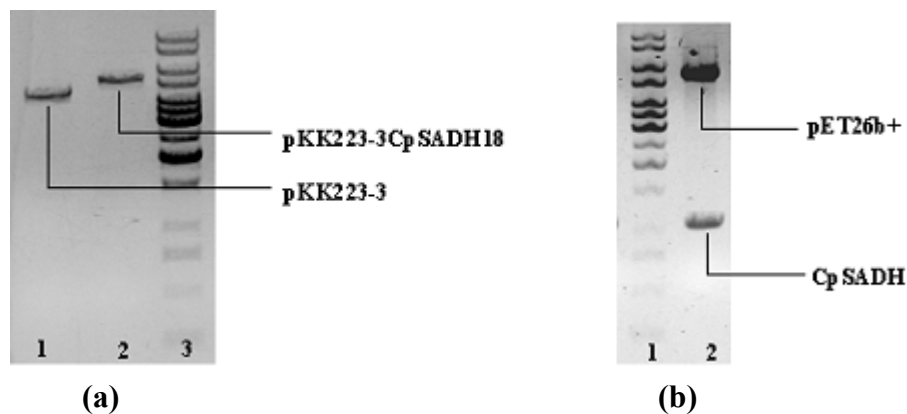
The cloning process was the same as described for PCR. The self-digested CpSADH and the expression vector pKK223-3 were isolated in each case after incubation with EcoRI and HindIII and ran on agarose gels to check for successful digestion. In case of pET-26b(+) expression vector, the CpSADH as well as the vector were digested in each case with NdeI and XhoI and later ran on agarose gels. A typical self-digested CpSADH and the individual vectors as represented by agarose gels are shown in the following two figures (Figure 17 a and b).

In order to check for positive clones and for successful ligation, the recombinant plasmids were isolated from the transformed bacteria and likewise digested with the same restriction enzymes and later verified by agarose gels. The positive clones were identified as plasmids with an increase in the total size due to the ligation of the CpSADH gene into the vectors. An alternative way to identify positive clones was to double digest the recombinant plasmids. By doing so, the CpSADH gene separated out of the pET-26b(+) vector, which was verified by an agarose gel. The recombinant constructs after restriction digestion are shown in Figure 18 (a) for recombinant vector construct pKK223-3CpSADH18 and in Figure 18(b) for the recombinant vector construct pET26b+CpSADH28his. The recombinant plasmids were further sequenced to check for the correctness of the CpSADH gene on the molecular level.

**Figure 17**

(a): Restriction digestion of the PCR amplified CpSADH gene and the expression vector pKK223-3. Lane 1= Digested pKK223-3 with EcoRI and HindIII for 4 h at 37 °C; Lane 2= Digested CpSADH fragment with EcoRI and HindIII for 4 h at 37 °C; Lane 3 = GeneRuler™ 1 kb DNA ladder.

(b): Restriction digestion of the PCR amplified CpSADH gene and the expression vector pET-26b(+). Lane 1= GeneRuler™ 1 kb DNA ladder; Lane 2= Digested pET-26b(+) with NdeI and XhoI for 16 h at 37 °C; Lane 3 = Digested CpSADH fragment with NdeI and XhoI for 16 h at 37 °C.

**Figure 18**

(a): Restriction digestion of the CpSADH/pKK223-3 recombinant vector construct after successful ligation and cloning. Lane 1= Digested pKK223-3 vector with EcoRI for 4 h at 37 °C as an example of self-ligation possessing no insert within it; Lane 2 = Digested pKK223-3CpSADH18 construct with EcoRI for 4 h at 37 °C; Lane 3 = GeneRuler™ 1 kb DNA ladder.

(b): Restriction digestion of the CpSADH/pET-26b(+) recombinant vector construct after successful ligation and cloning. Lane 1= GeneRuler™ 1 kb DNA ladder; Lane 2 = Digested pET26b+CpSADH28his construct with NdeI and XhoI for 4 h at 37 °C. The double digestion separated the CpSADH gene from the pET-26b(+) vector as an evidence of successful ligation.

It was observed that with proteins from *C.albicans*, on translation level, for the triplet codon CTG a serine instead of a leucine could be inserted (Kawaguchi et al., 1989; Suzuki et al., 1993; Santos et al., 1993). This deviation from the universal genetic code and/or these not standardized translation events could lead to the change of the enzyme characteristics and/or to the shift of the open reading frame (ORF). Such a triplet was not found in the determined sequence of recombinant CPCP in this work and also not in Bönitz's.

The DNA sequencing of the CpSADH18 (recombinant CpSADH without His₆-tag) and CpSADH28his (recombinant CpSADH with His₆-tag) that had been isolated from the DraI gDNA library of *C.parapsilosis* DSMZ 70125 strain showed that all the amino acids of the patented ADH from *C.parapsilosis* IFO 1396 strain were conserved. But an interesting thing that had been noticed through this sequencing was that at position 58 of the DNA sequence of the patented ADH (Kojima et al., 1995) a triplet codon CTG (coding for leucine) is present. Fortunately, in the CpSADH gene from *C.parapsilosis* DSMZ 70125, a triplet codon TTG (also coding for leucine) was present instead of the CTG. The possibility of “wobble bases” due to PCR could be ruled out in this case because both the CpSADH18 that has been amplified with the ISIS & GOTM Mastermix and the CpSADH28his that has been amplified with TripleMaster[®] PCR System, conserved this triplet codon TTG. The alignment of the DNA sequences of all the three types of the CpSADH genes is shown in the following figure.

patented CpSADH	-----ATGTCAATTCCATCAAGCCAGTACGGATTTCGTATTCAAT
recCpSADH	-----ATGTCAATTCCATCAAGCCAGTACGGATTTCGTATTCAAT
CpSADH-NHis	ATGCACCATCATCATCACCACATGTCAATTCCATCAAGCCAGTACGGATTTCGTATTCAAT

patented CpSADH	AAGCAATCAGGACTTAATTGAGAAATGATTGCTGTCCACAAGCCCAAAGCGGGTCAA
recCpSADH	AAGCAATCAGGACTTAATTGAGAAATGATTGCTGTCCACAAGCCCAAAGCGGGTCAA
CpSADH-NHis	AAGCAATCAGGACTTAATTGAGAAATGATTGCTGTCCACAAGCCCAAAGCGGGTCAA

patented CpSADH	TTGTTGTTGAAAGTTGATGCTGTTGGATTGTGTCATTCTGATTACATGTCATTTACGAA
recCpSADH	TTGTTGTTGAAAGTTGATGCTGTTGGATTGTGTCATTCTGATTACATGTCATTTACGAA
CpSADH-NHis	TTGTTGTTGAAAGTTGATGCTGTTGGATTGTGTCATTCTGATTACATGTCATTTACGAA

patented CpSADH	GGGTTGGATTGTGGTGATAATTATGTCATGGGACATGAAATTGCTGGAAGTGTGCTGCT
recCpSADH	GGGTTGGATTGTGGTGATAATTATGTCATGGGACATGAAATTGCTGGAAGTGTGCTGCT
CpSADH-NHis	GGGTTGGATTGTGGTGATAATTATGTCATGGGACATGAAATTGCTGGAAGTGTGCTGCT

patented CpSADH	GTGGGTGATGATGTCATTAAGTACAAGGTTGGTGATCGTGTGCTGTGTCGGACCCAAT
recCpSADH	GTGGGTGATGATGTCATTAAGTACAAGGTTGGTGATCGTGTGCTGTGTCGGACCCAAT
CpSADH-NHis	GTGGGTGATGATGTCATTAAGTACAAGGTTGGTGATCGTGTGCTGTGTCGGACCCAAT

patented CpSADH	GGATGTGGTGGGTGCAAGTATTGTCGTGGTGCCATTGACAATGTATGTAACGCAATTT
recCpSADH	GGATGTGGTGGGTGCAAGTATTGTCGTGGTGCCATTGACAATGTATGTAACGCAATTT
CpSADH-NHis	GGATGTGGTGGGTGCAAGTATTGTCGTGGTGCCATTGACAATGTATGTAACGCAATTT

patented CpSADH	GGTGATTGGTTCGGATTGGGGTACGATGGTGGGTATCAACAGTACTTGTGGTTACTAGA
recCpSADH	GGTGATTGGTTCGGATTGGGGTACGATGGTGGGTATCAACAGTACTTGTGGTTACTAGA
CpSADH-NHis	GGTGATTGGTTCGGATTGGGGTACGATGGTGGGTATCAACAGTACTTGTGGTTACTAGA *****
patented CpSADH	CCACGTAACCTTGTCTCGTATCCCAGATAACGTATCTGCAGACGTGGCTGCGGCTTCAACT
recCpSADH	CCACGTAACCTTGTCTCGTATCCCAGATAACGTATCTGCAGACGTGGCTGCGGCTTCAACT
CpSADH-NHis	CCACGTAACCTTGTCTCGTATCCCAGATAACGTATCTGCAGACGTGGCTGCGGCTTCAACT *****
patented CpSADH	GATGCTGTATTGACACCATATCACGCAATCAAGATGGCTCAAGTGTACCAACTTCGAAT
recCpSADH	GATGCTGTATTGACACCATATCACGCAATCAAGATGGCTCAAGTGTACCAACTTCGAAT
CpSADH-NHis	GATGCTGTATTGACACCATATCACGCAATCAAGATGGCTCAAGTGTACCAACTTCGAAT *****
patented CpSADH	ATCTTGCTTATTGGTGCTGGTGGATTGGGTGGAAATGCAATTCAAGTTGCCAAGGCATTT
recCpSADH	ATCTTGCTTATTGGTGCTGGTGGATTGGGTGGAAATGCAATTCAAGTTGCCAAGGCATTT
CpSADH-NHis	ATCTTGCTTATTGGTGCTGGTGGATTGGGTGGAAATGCAATTCAAGTTGCCAAGGCATTT *****
patented CpSADH	GGTGCGAAAGTTACTGTTTTGGACAAAAAAAGGAGGCTCGTGACCAAGCAAAGAAGTTG
recCpSADH	GGTGCGAAAGTTACTGTTTTGGACAAAAAAAGGAGGCTCGTGACCAAGCAAAGAAGTTG
CpSADH-NHis	GGTGCGAAAGTTACTGTTTTGGACAAAAAAAGGAGGCTCGTGACCAAGCAAAGAAGTTG *****
patented CpSADH	GGTGCTGATGCAGTTTATGAAACATTGCCAGAATCCATTTCTCCTGGCTCTTTTTTCAGCA
recCpSADH	GGTGCTGATGCAGTTTATGAAACATTGCCAGAATCCATTTCTCCTGGCTCTTTTTTCAGCA
CpSADH-NHis	GGTGCTGATGCAGTTTATGAAACATTGCCAGAATCCATTTCTCCTGGCTCTTTTTTCAGCA *****
patented CpSADH	TGTTTTGATTTTGTTCAGTGCAAGCTACATTTGATGTATGTCAAAAGTATGTTGAACCA
recCpSADH	TGTTTTGATTTTGTTCAGTGCAAGCTACATTTGATGTATGTCAAAAGTATGTTGAACCA
CpSADH-NHis	TGTTTTGATTTTGTTCAGTGCAAGCTACATTTGATGTATGTCAAAAGTATGTTGAACCA *****
patented CpSADH	AAGGGTGTAATTATGCCCGTGGGACTCGGTGCTCCTAATTTATCGTTTAATTTGGGAGAT
recCpSADH	AAGGGTGTAATTATGCCCGTGGGACTCGGTGCTCCTAATTTATCGTTTAATTTGGGAGAT
CpSADH-NHis	AAGGGTGTAATTATGCCCGTGGGACTCGGTGCTCCTAATTTATCGTTTAATTTGGGAGAT *****
patented CpSADH	TTGGCATTGAGAGAAATTCGAATCTTGGGTAGTTTTTGGGGAAC TACTAATGATTGGAT
recCpSADH	TTGGCATTGAGAGAAATTCGAATCTTGGGTAGTTTTTGGGGAAC TACTAATGATTGGAT
CpSADH-NHis	TTGGCATTGAGAGAAATTCGAATCTTGGGTAGTTTTTGGGGAAC TACTAATGATTGGAT *****
patented CpSADH	GATGTTTTGAAATTGGTTAGTGAAGGTAAAGTTAAACCCGTTGTGAGAAGTGCCAAATTG
recCpSADH	GATGTTTTGAAATTGGTTAGTGAAGGTAAAGTTAAACCCGTTGTGAGAAGTGCCAAATTG
CpSADH-NHis	GATGTTTTGAAATTGGTTAGTGAAGGTAAAGTTAAACCCGTTGTGAGAAGTGCCAAATTG *****
patented CpSADH	AAGGAATTGCCAGAGTATATTGAAAAATTGAGAAACAATGCTTATGAAGGTAGAGTTGTT
recCpSADH	AAGGAATTGCCAGAGTATATTGAAAAATTGAGAAACAATGCTTATGAAGGTAGAGTTGTT
CpSADH-NHis	AAGGAATTGCCAGAGTATATTGAAAAATTGAGAAACAATGCTTATGAAGGTAGAGTTGTT *****
patented CpSADH	TTTAATCCATAG
recCpSADH	TTTAATCCATAG
CpSADH-NHis	TTTAATCCATAG *****

Figure 19: Global alignment score of the patented CpSADH with that of the CpSADH's that have been isolated in this work. The “*” designates similarities with the patented CpSADH. The exchange of “CTG” with “TTG” is represented with the grey-marked region.

Using the **NetNGlyc 1.0** server that predicts N-glycosylation sites in human protein, N-linked glycosylation motifs of N-L-S at position 136, N-V-S at position 143 and N-L-S at position 266 were found to be present in the amino acids sequences of the patented CpSADH by Kojima (1995) as well as in the recombinant CpSADH that had been cloned in this work. But as stated earlier, such motifs are not sufficient for glycosylation of proteins that do not contain signal peptides. Analysis of the amino acids sequence of CpSADH with the **SignalP** server showed that the CpSADH contained no signal peptides within it. Therefore the possibility of real glycosylation of CpSADH could be ruled out. Besides Kojima reported also of no such probabilities and the enzyme was active when expressed in *E.coli*.

```

1  msipssqygf vfnkqsglnl rndlpvhkpk agqlllkvda vglchsdhvh iyegldcgn
61  yvmgheiagt vaavgddvin ykvgdrvacv gpngcggcky crgaidnvck nafgdwfglg
121 ydgggyqqyll vtrprnlstri pdnvsadvaa astdavltpy haikmaqvsp tsnilligag
181 glggnaiqva kafgakvtvl dkkkeardqa kklgadavye tlpesispgs fsacfdvsv
241 qatfdvcqky vepkgvimpv glgapnlsfn lgdlalreir ilgsfwgtn dlddvklvs
301 egkvkpvvrs aklkelpeyi eklrnnayeg rvvfnp Stop (336 amino acids)

```

Figure 20: Translated amino acids sequence of CpSADH.

3.1.7 Conclusion of the cloning of CPCr and CpSADH

The CPCr and the CpSADH had been successfully cloned. Using the method of genome walking and the partial amino acids sequences of the wtCPCr, the CPCr gene was amplified from the gDNA library of *C.parapsilosis* DSMZ 70125. The recombinant CPCr possessed 403 amino acids, which were 54 amino acids more on the N-terminal when compared to the amino acids sequences of the recombinant CPCr that had been cloned by Bönitz (2001). But the CPCr (derived in this work) possessed all the known partial amino acids sequences within it. A reason behind this lack of 54 amino acids on the N-terminal of the CPCr cloned by Bönitz could be that the sequence somehow got detached from the rest of the sequence and was not identifiable in the partial amino acids sequences of the wtCPCr and thus this sequence began with a proline as a start codon which was quite unusual. These 54 amino acids began with a methionine as a start codon and were on the same reading frame with the rest of the sequences (as described by Bönitz). This therefore confirmed that the 403 amino acids of the CPCr derived in this work had a potential start codon methionine and a stop codon TAA and was thus a complete amino acids sequence. Analysis of the 403 amino acids of the recombinant CPCr showed that it contained no signal peptides within the first 54

amino acids on the N-terminal and that they were parts of the mature protein. Thus with the method of genome walking the complete sequence of the CPCr was derived.

The CpSADH gene coding for an alcohol dehydrogenase that had been patented by Kojima in 1995 were also amplified from the same gDNA library of *C.parapsilosis* DSMZ 70125. The identities of these two enzymes (CpSADH derived in this work and the patented enzyme) when compared with the amino acids were 100%. But on the DNA level, the patented ADH from *C.parapsilosis* IFO 1396 strain had a “CTG” triplet at position 58 whereas the recombinant CpSADH from *C.parapsilosis* DSMZ 70125 strain had a “TTG” triplet instead. Though both the triplet codons codes for leucine (according to the universal genetic code) but it had been reported that a “CTG” in *C.albicans* coded for serine instead. Such a replacement of serine with leucine could alter the enzyme characteristics or activity when expressed in *E.coli*. A similar triplet codon was not found in the DNA sequence of the recombinant CPCr. Amplification of both the CPCr and the CpSADH genes from the same strain (DSMZ 70125) of *C.parapsilosis* which is known to be the “CPCr” type strain confirmed that at least two alcohol dehydrogenases were present in the same strain though it was not clear which one of the two is the “Real CPCr”.

According to Reid (Reid and Fewson, 1994) the NAD- or NADP-dependent alcohol dehydrogenases can be classified into three groups depending in their number of amino acids. These include the Group I long-chain (also known as medium-chain) (approximately 350 amino acid residues) zinc-dependent alcohol dehydrogenases, Group II short-chain (approximately 250 amino acid residues) zinc-independent alcohol dehydrogenases and the Group III (also known as long-chain) “iron-activated” alcohol dehydrogenases that generally contain approximately 385 amino acid residues or even up to 900 residues. Accordingly, the recombinant CPCr that had been cloned in this work can be classified as a long-chain ADH (Group III) since the sequence consisted of 403 amino acids. Both the recombinant CPCr from Bönitz’s work that consisted of only 349 amino acids, and the CpSADH that consisted of 336 amino acid residues can be categorized therefore as medium-chain ADH (Group I).

3.2 Expression of recombinant CPCR and CpSADH

Conditions for the recombinant expression of genes

The highest goal of a gene cloning for biotechnological applications is the expression of the cloned gene in a selected host organism, so that the target protein is produced in sufficiently large quantities for commercial employment. Different molecular-biological characteristics are used for the regulation of gene expression:

1. The conditions of necessary promoter and terminator sequences for transcription.
2. The strength of the ribosome-binding site for the initiation of translation.
3. The copy number of the cloned genes in the host organism and whether a gene is placed on a plasmid or is integrated in the host genome.
4. The final cellular position of the synthesized foreign protein.
5. The efficiency of translation in the host organism.
6. The intrinsic stability of the coded protein in the host cell (Glick, 1987; Otha, 1993; Glick, 2003).

Other parameters that also are to be considered are the possibilities for the purification of the expressed protein from the host organism, and the possibilities for cultivation of huge cell mass through fermentation. These conditions are mainly necessary in order to make the recombinant protein available for industrial applications, as the primary goals of recombinant proteins from the industrial point of view are high product yield, stability and cost effectiveness.

The intensity of expression of a foreign protein depends also on the characteristics of the host organism. Although many prokaryotic and eukaryotic organisms like *Bacillus subtilis*, different yeasts such as *Saccharomyces cerevisiae*, *Hansenula polymorpha* or *Pichia pastoris*, or also animal cells, plants cells, and insects (Wilcox and Studnicka, 1988) can be used as host systems for the heterologous expression of genes, one uses *E.coli* for most commercially important, genetically modified proteins, at present. The reason behind this is that the physiology, molecular biology and genetics of this bacterium are very well known. Furthermore, this gram-negative bacterium in contrary to other microorganisms can be produced with simple media in large scale and many proteins can be very fast and economically experimented.

One way for achieving the expression of recombinant proteins is with the plasmid coding system because this system allows high product yield. The expression plasmids possess a promoter region for initiation of transcription of the gene, a terminator sequence for transcription stop at the end of the gene sequence. The expression plasmids contain at least one selection marker. An “Origin of replication” ensures that a plasmid is multiplied independently of the bacterial chromosome (Dale, 1994). Numerous systems possess additional DNA sequences to reduce problems such as mRNA or plasmid stability, premature transcription termination or an ineffective translation initiation (Balbas and Bolivar, 1990).

3.2.1 Expression of recombinant CPCr

The recombinant plasmid constructs carrying the gene coding for CPCr namely pKK223-3CPCr9 and pET26b+CPCr9his that had been created by successful cloning as described in section (3.1.5) were used for the expression of CPCr. For carrying out expression of the recombinant CPCr in *E.coli*, the plasmid pKK223-3CPCr9 was transformed into the *E.coli* JM109 strain and the plasmid pET26b+CPCr9his was transformed into BL21(DE3) strain. The expression systems were therefore termed as JM109pKK223-3CPCr9 and BL21(DE3)pET26b+CPCr9his, respectively. The vector maps of the expression plasmids are shown in Figure 21 that had been created using the computer program CloneManager 6.0 (Scientific & Educational Software, USA).

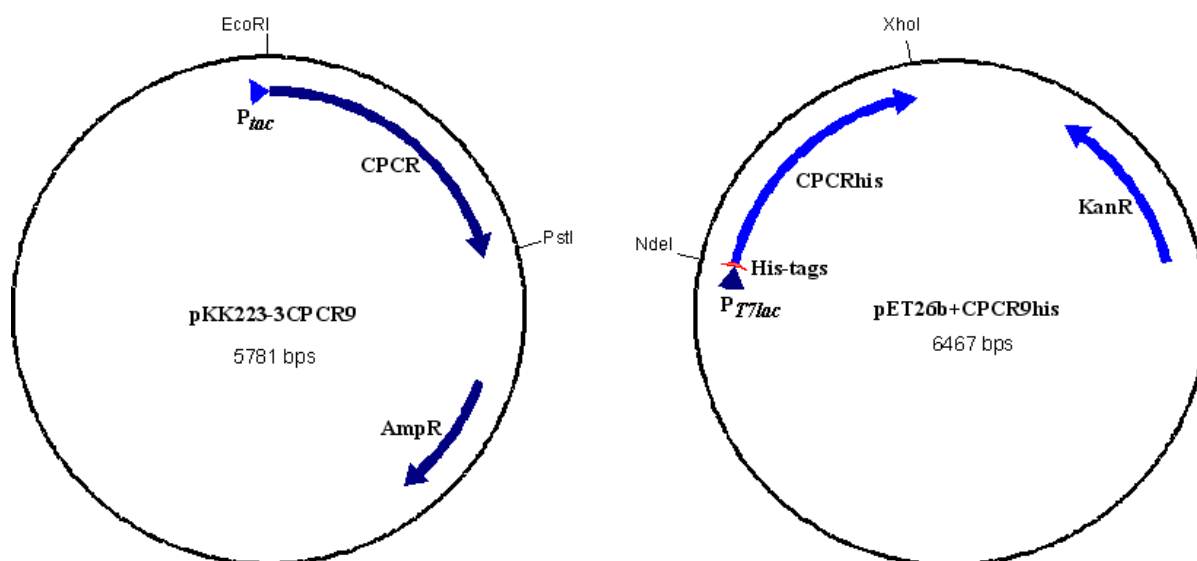


Figure 21: Construction of expression plasmids pKK223-3CPCr9 and pET26b+CPCr9his.

The recombinant CPCR was expressed with the methods as described earlier (see section 2.2.2) in 100-200 ml scales. Cell free crude extracts of these recombinant strains were used to determine the specific activity of the expressed recombinant CPCR with the standard assay conditions. As a negative control, the same *E.coli* strains JM109 and BL21(DE3) were transformed with the parent vector alone (i.e. pKK223-3 and pET-26b+ respectively) and were expressed in parallel with the same conditions and afterwards cell free crude extracts were prepared in the same way like that with the enzymes. It was found that when determining the activities of the crude extracts by measuring the oxidation of $\text{NADH} + \text{H}^+$ with the spectrophotometer, there were always some background activities of the negative controls. Therefore the specific activities of the negative controls were used as reference. These values were always subtracted from the values obtained with the enzymes. In her work, Bönitz reported to have a specific activity of recombinant CPCR of only 0.3 U/mg with the pKK223-3/*E.coli* HB101 construct, no activity with the pKK223-3/*E.coli* JM109 construct, and a maximum of 1.5 U/mg with the pBTac/*E.coli* JM105 construct when fermentation was carried out in 100 ml scales. In comparison, the specific activities of the crude extracts of the recombinant constructs JM109pKK223-3CPCR9 was 0.4 U/mg and that of the BL21(DE3)pET26b+CPCR9 was 0.5 U/mg when fermentation was done in 100 ml scales in shake flasks with LB medium at 30 °C. Induction was done with 1 mM IPTG and after induction, cells were grown at 30 °C for the pKK223-3/*E.coli* JM109 construct and at 28 °C for the pET26b+/*E.coli* BL21(DE3) construct for 6 h.

An interesting observation found with the recombinant CPCR both with and without His₆-tag, that had been obtained in this work was that the recombinant protein was expressed in the host strains only for about 2-3 times. Typically, after the 2nd expression, the enzyme was not active anymore. This was noticed for both the expression systems. Alteration in the expression parameters like temperature (from 30 °C to as low as 18 °C), IPTG concentrations (from 1 mM to 0.1 mM), media compositions, and period of induction all resulted in no expression. Analysis of the DNA sequences showed no point mutations or alterations of amino acids within the sequences when compared with the DNA sequence of the CPCR derived by genome walking. One reason could be instability of the plasmids within the host organisms due to storage. The expression plasmids were therefore transformed freshly and were then used for expression with induction of IPTG. But, the enzyme was not expressed at all. Toxicity of the CPCR to the host strains could be ruled out because the recombinant *E.coli* cells grew normally under the expression conditions and was not inhibited upon induction. To

avoid proteolytic degradation, protease inhibitor such as PMSF were also added but gave no positive results. Many recombinant proteins form insoluble aggregates known as inclusion bodies when over-expressed in *E.coli* (Rudolf and Lilie, 1996; Clark 1998; Lilie et al., 1998). The recombinant CpSADH was checked for such inclusion bodies by making SDS-PAGE analysis of the expressed bacterial cell pellets and that as well gave no positive signals.

3.2.2 Expression of recombinant CpSADH

The expression of the recombinant CpSADH was carried out using the expression plasmids pKK223-3CpSADH18 and pET26b+CpSADH28his that had been successfully cloned as described in section (3.1.6). The recombinant CpSADH was expressed in *E.coli* strains. For that, the plasmid pKK223-3CpSADH18 was transformed in competent *E.coli* JM109 and the plasmid pET26b+CpSADH28his was transformed in competent *E.coli* BL21(DE3) strains respectively. The recombinant expression strains thereby created namely, JM109 pKK223-3CpSADH18 contained the CpSADH without any His₆-tag whereas BL21(DE3) pET26b+CpSADH28his contained the CpSADH with an N-terminal His₆-tag. The vector maps of the expression plasmids are shown in the following figure:

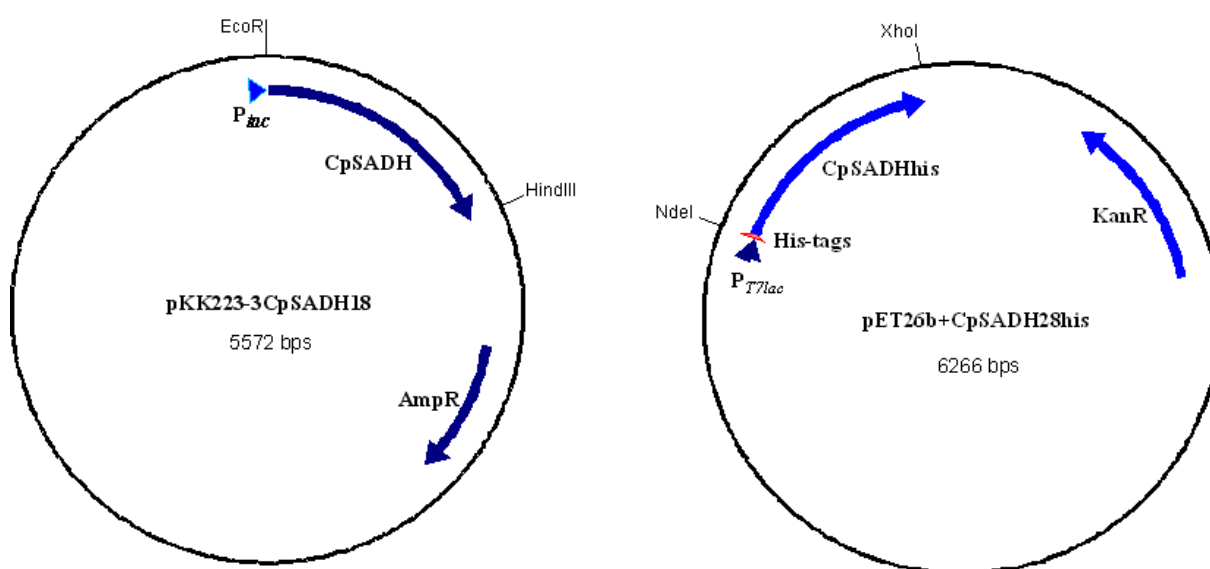


Figure 22: Construction of expression plasmids pKK223-3CpSADH18 and pET26b+CpSADH28his.

Expression of the recombinant CpSADH was carried out in a volume ranging from 100 ml to 4 liters scales with the standard method for expression as described previously (see section

2.2.2). Cell free cleared lysates of these recombinant strains were used to determine the activity, specific activity of the expressed recombinant CpSADH using the standard assay conditions. As a negative control, results of similar activity assay of the host *E.coli* cells JM109 and BL21(DE3) that were transformed with the vectors pKK223-3 and pET-26b(+) respectively were used for reference. For the expression of CpSADH, batch fermentation was carried out in 1 liter scale in TB medium in shake flasks. Cells were induced with 1 mM IPTG and were grown for an additional 6 h at 30 °C (JM109pKK223-3CpSADH18) and at 28 °C [BL21(DE3)pET26b+CpSADH28his]. Under these conditions, the specific activities of the recombinant CpSADH in these two expression systems thereby measured were 14.97 U/mg with the construct JM109pKK223-3CpSADH18 whereas 15.3 U/mg with the construct BL21(DE3)pET26b+CpSADH28his.

The values of the specific activities of the crude extracts of the two expression systems of CpSADH were obtained under the optimized conditions for expression. It was found that for the recombinant expression strain JM109pKK223-3CpSADH18 that contained the CpSADH gene without any His₆-tag, induction with 1 mM IPTG in the mid-log phase (OD₅₅₀ of ~1.6) resulted in comparatively higher activities/specific activities than when induced at early mid-log phase (OD₅₅₀ of ~1.2). The recombinant strain BL21(DE3)pET26b+CpSADH28his was always induced at an OD₆₀₀ of 0.8. For the pET expression systems, this OD was described the best for the expression of recombinant proteins. These results showed that addition of an N-terminal His₆-tag on the CpSADH did not alter the activities of the protein. Besides it was found that the specific activity of the His-tagged CpSADH was slightly higher than that of the CpSADH without tag.

Fermentation of recombinant CpSADH

Fermentation of the recombinant expression strains was carried out in 100 ml to 4 liters scales. For preparative scales, batch-fermentation of the expression strains JM109pKK223-3CpSADH18 and BL21(DE3)pET26b+CpSADH28his were carried out in 5-liter shake flasks with baffles with culture media of 1 liter in each flask.

The level of expression of the recombinant proteins depends on the composition of the growth media (Broedel et al., 2001). It was found that the expression of recombinant CpSADH was also influenced by the components of the media. Therefore, LB and TB

mediums were used to find out the best-optimized media composition. It was found that under the same expression conditions (here: 100 ml growth media, growth temperature at 30 °C and induction with 1 mM IPTG), the recombinant CpSADH showed specific activities of 4.5 U/mg when grown in LB medium and 5.5 U/mg when grown in TB medium.

TB medium like other rich nutrient medium has a higher amount of yeast extract and also glycerol, which serves as a carbon source. Fermentation with TB medium resulted in higher amount of biomass of the expressed *E.coli* strain as well as showed the highest level of protein expression. TB medium was therefore used for preparative fermentation of both JM109pKK223-3CpSADH18 and BL21(DE3)pET26b+CpSADH28his expression strains for further enzyme production.

Fermentation of the expression strain JM109pKK223-3CpSADH18 was carried out in 5-liter shake flasks with baffles with the optimized fermentation conditions as described earlier in this section, like growth at 30 °C, induction with 1 mM IPTG at mid-log phase and using TB medium. Under these conditions, it was found that from 4 liters of fermentation as much as 40 gram of biomass was achieved. In case of the expression strain BL21(DE3)pET26b+CpSADH28his, under the optimized conditions of fermentation like growth at 30 °C using TB medium, induction with 1 mM IPTG at OD₆₀₀ of 0.8, followed by temperature decrease of 28 °C, also carried out in 5-liter shake flasks with baffles, resulted in a biomass of 28 gram from 4 liters of fermentation. The amount of biomass achieved in this instance was much less when compared to JM109pKK223-3CpSADH18. One reason could be the decrease of temperature from 30 °C to 28 °C (after induction), which in turn decreased the overall metabolism of the bacteria and increased the overall expression level of the recombinant protein (Vasina and Baneyx, 1996; Schirano and Shibata, 1990; Schein and Noteborn, 1988). This was clear from the total units of enzyme that was recovered from each of the fermentations. The total biomass yield and the total enzyme produced from each of the fermentations are summarized in Table 3.

	JM109pKK223-3CpSADH18	BL21(DE3)pET26b+CpSADH28his
Total Biomass yield	40 gram from 4 liters	28 gram from 4 liters
Total Enzyme yield	20632 units from 4 liters	20845 units from 4 liters

Table 3: Total biomass and enzyme yields obtained from 4 liters of cultivation medium through batch-fermentation in shake flasks with baffles.

As can be seen from Table 3, as much as 10 g biomass/l with a corresponding CpSADH total activity of 5158 U/l was obtained from the expression strain JM109pKK223-3CpSADH18. In the case of the expression strain BL21(DE3)pET26b+CpSADH28his, 7 g biomass/l with a total enzymatic activity of 5211 U/l was obtained. The total activities per liter fermentation media that are stated here for both the expression strains, are much higher than that described by Bönitz (2001), where she stated that a maximum total activity of only 343 U/l fermentation media was achieved by the expression of recombinant CPCR. The specific activity of the crude extracts of the recombinant CpSADH (without His₆-tag) was 15 U/mg and that of CpSADH (with His₆-tag) was 15.3 U/mg. Comparatively, Bönitz reported that the maximum specific activity of the crude extract of recombinant CPCR obtained was 2.5 U/mg (from 20 liters batch-fermentation). The specific activity of the crude extract of the recombinant CpSADH (Kojima et al., 1995) was described to be as low as 0.6 U/mg.

Purification of recombinant CpSADH (JM109pKK223-3CpSADH18 construct)

Cell free cleared lysate of the recombinant CpSADH (without His₆-tag) was used for the purification of the recombinant protein through anion-exchange chromatography (Macro Prep High Q). The elution of the bound proteins was made by step gradients. The first fractions were the cations that were not bound to the matrix at all. The relatively weak ionic bound proteins were eluted with 100 mM sodium chloride. CpSADH was eluted with a salt concentration from 200 mM onwards. The active fractions showing the activity for CpSADH were analyzed with an SDS-PAGE to determine the quality of the purified protein. The elution profile of the purification steps as well as the SDS-PAGE of the active fractions is shown in Figure 23(a) and (b) respectively.

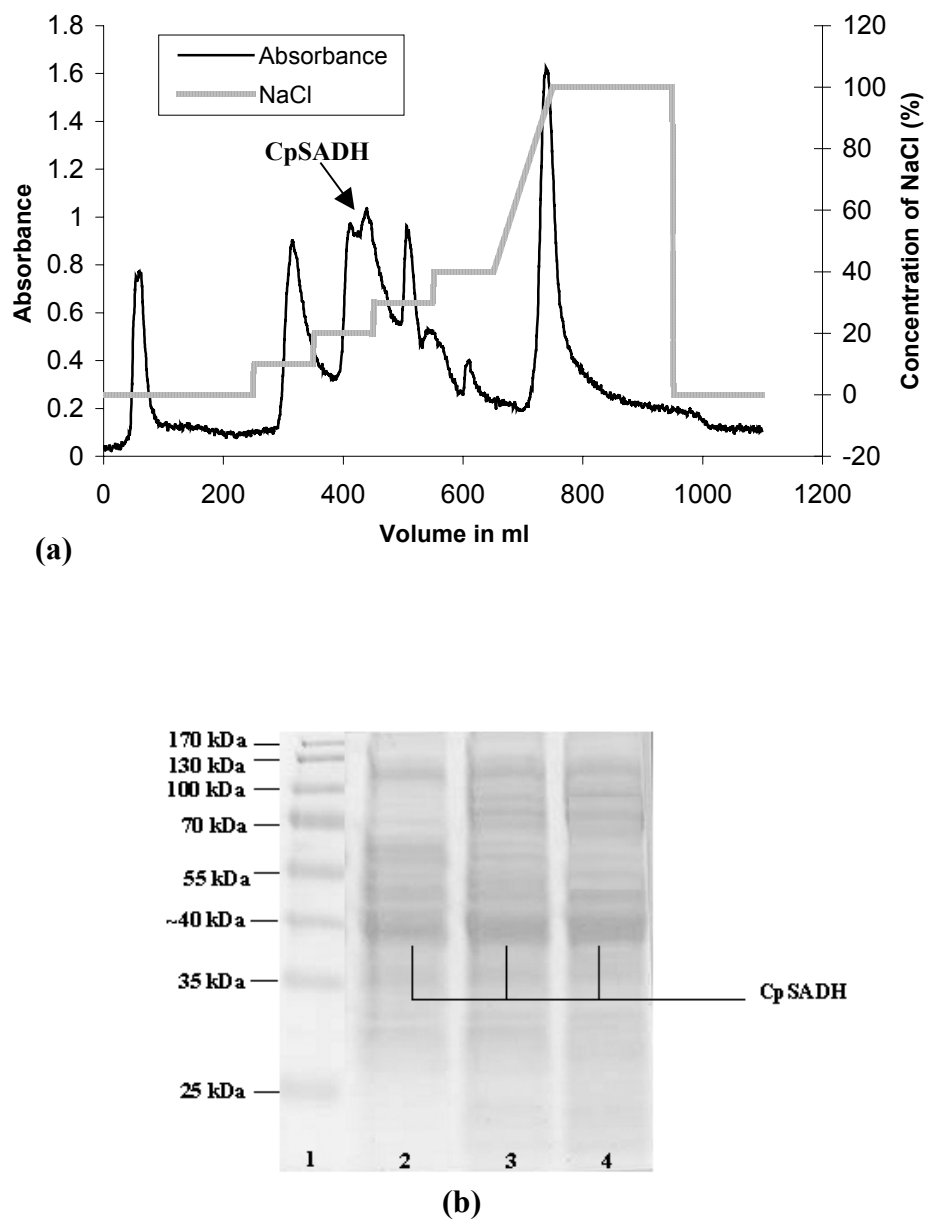


Figure 23

(a): Elution profile of the purification steps of recombinant CpSADH (without His₆-tag) through anion-exchange chromatography with step-gradient.

(b): SDS-PAGE analysis of the active fractions of purified CpSADH. Lane 1= PageRuler™ Prestained Protein Ladder. Lanes 2,3 and 4 = Eluted active fractions of CpSADH after anion-exchange chromatography.

The CpSADH was eluted together with other proteins, which was clear from the pattern of peak that was achieved. Further the SDS-PAGE also showed the presence of other proteins together with the purified CpSADH. The size of the CpSADH determined by the SDS-PAGE

analysis was approximately 40 kDa, the same as stated by Kojima (1995). A disadvantage of such a matrix is that all proteins that were anions were also bound and with an increase in salt concentration were eluted together with CpSADH. A continuous linear gradient instead of step gradient was also tried in order to purify the CpSADH through the anion-exchange support. Most of the weakly bound proteins were eluted at 100 mM sodium chloride concentration. A continuous gradient was followed thereafter from 100-500 mM NaCl. Fractions showing the activity of CpSADH were eluted from about 200 mM onwards and went up to about 270 mM of NaCl. The elution profile is shown in the following figure:

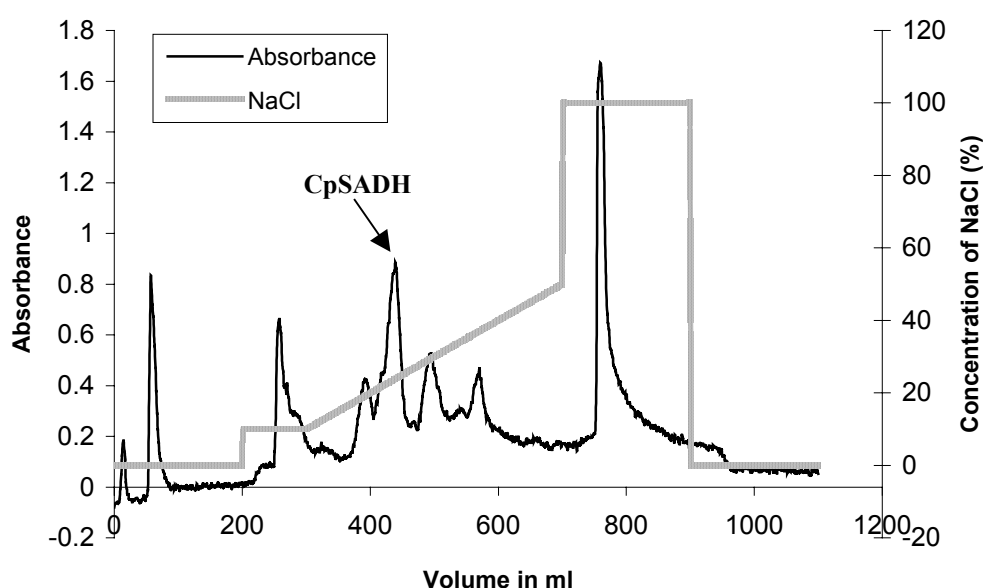


Figure 24: Elution profile of the purification steps of recombinant CpSADH (without His₆-tag) through anion-exchange chromatography with continuous linear-gradient.

The analysis of the eluted active fractions on SDS-gel showed the presence of other proteins as well, and the pattern was more or less the same as had been shown in Figure 23 (b). The active fractions of CpSADH were concentrated and also desalted through ultrafiltration (MWCO 10000 Da). The concentrated and desalted partially purified CpSADH was then lyophilized and the enzyme powder was stored at -20°C till use. An SDS-gel picture of the CpSADH after ultrafiltration is shown in Figure 25. The purification data of the CpSADH are described in Table 4.

Purification Steps	Enzyme (Units)	Protein (mg)	Volume of fraction (ml)	Yield (%)	Specific Activity (U/mg)	Purification Factor (Enrichment)
Crude Extract	1800	200	16	100	9	1
Anion-Exchange Chromatography (Macro Prep High Q)	996	10.5	60	55.3	94.9	10.5
Ultrafiltration 10000 Daltons Cut-off	897	9.2	6	49.8	97.5	10.8

Table 4: Purification data of CpSADH with Macro Prep High Q matrix.

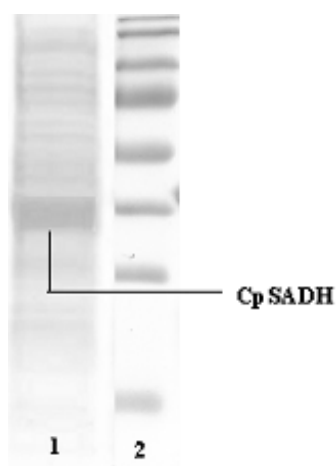


Figure 25: SDS-PAGE analysis of the purified CpSADH after ultrafiltration. Lane 1= Concentrated and purified CpSADH after anion-exchange chromatography; Lane 2 = PageRuler™ Prestained Protein Ladder.

The specific activity of the recombinant CpSADH purified through anion-exchange chromatography increased from 9 U/mg to 94.9 U/mg and after ultrafiltration reached to 97.5 U/mg. A yield of only 49.8% was finally achieved. The enrichment factor reached about 10.8 fold. Using Q-Sepharose FF matrix, Bönitz reported that the recombinant CPCR reached a specific activity of 32.1 U/mg and a purification factor of 13.9. Thus the specific activity of CpSADH after anion-exchange chromatography was much higher than that of Bönitz's (after Q-Sepharose).

Purification of recombinant CpSADH with His₆-tag [BL21(DE3)pET26b+CpSADH28 construct]

For industrial applications, recombinant proteins with higher specific activities, higher yield and with fast & easy purification conditions are favored. Enzymes with His₆-tags are widely known for their easy one-step purification conditions and usually with high yields and very high specificities and very low or no background proteins. This is usually done by introducing six histidine residues either on the N- or on the C-terminal of the protein. Such a modification of the N- or C-terminal with addition of only six amino acids is tolerable to many recombinant proteins and the likelihood that such a fusion would adversely affect gene function is reduced (Lesley, 2001). But many recombinant proteins are not active anymore or show changes in their conformation, specificities with the slightest change in their amino acids sequence. For such proteins, the histidine residues could be cleaved off from the main protein after purification by using several commercially available selective proteases. For the His-tagged CpSADH it was presumed that such histidine residues would not affect the folding of the protein and was used for purification under native conditions.

The cell free cleared lysate of the N-terminal His-tagged recombinant CpSADH was purified by Immobilized-Metal Affinity Chromatography (IMAC) using a Ni-Nitrotriactic Acid (Ni-NTA) superflow matrix (Qiagen). Cell free cleared lysate of the recombinant CpSADH (with His₆-tag) was used for the purification of the recombinant protein through Ni-NTA matrix. The elution of the bound proteins was made by an increase in the imidazole concentration. The first fractions eluted were the bacterial proteins that were not bound to the matrix at all and also some weakly bound proteins that were eluted with an imidazole concentration of 10 mM. His-tagged CpSADH was eluted with an increased imidazole concentration of 250 mM. The elution profile of the purification steps of CpSADH (His₆-tagged) is shown in Figure 26.

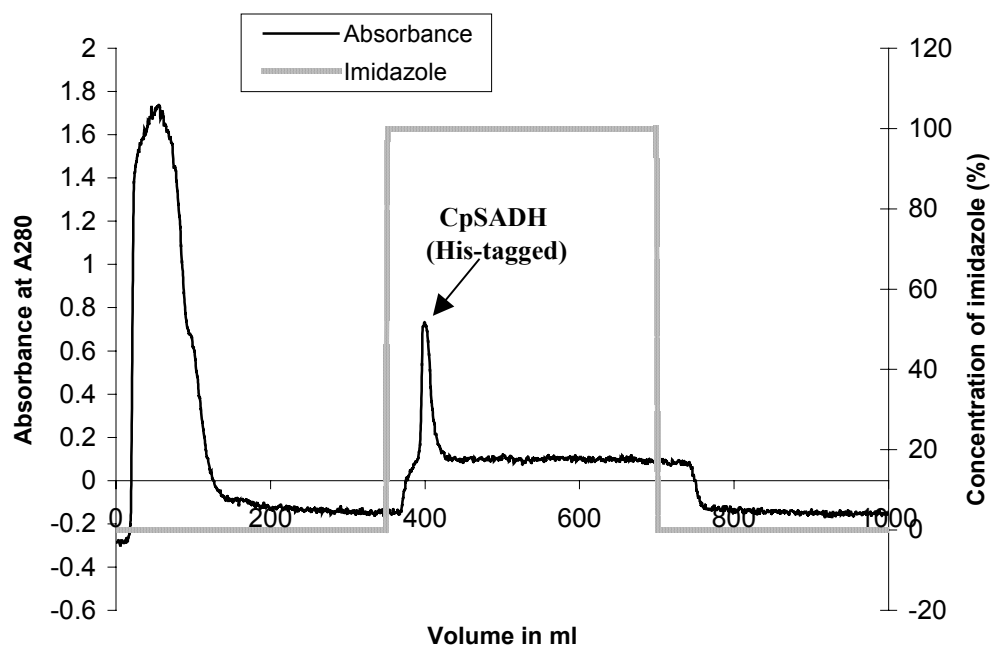


Figure 26: Elution profile of the purification steps of recombinant CpSADH (with His₆-tag) by IMAC using a Ni-NTA superflow matrix.

Increasing the concentration of imidazole, the recombinant His-tagged CpSADH was eluted very easily. The His-tagged CpSADH was not stable in imidazole (250 mM) and it was therefore necessary to desalt the active fractions after IMAC purification. This was done by ultrafiltration by an exchange of buffer (TEA-HCl, pH 7.5) in at least three cycles. After desalting, the final volume of the purified His-tagged CpSADH was reduced/concentrated to at least half of the initial volume of the active IMAC fractions. The concentrated and desalted purified CpSADH was then lyophilized and the enzyme powder was stored at -20°C . Alternatively, the enzyme could also be stored as liquid in 50% (v/v) glycerol at -20°C without any loss of activity for several months. The purification data of the His-tagged CpSADH is summarized in Table 5. To determine the quality of the purified recombinant protein, each of the fractions was analyzed with SDS-PAGE and also with Western blot, the results of which are shown in Figure 27 (a) and (b) respectively.

Purification Steps	Enzyme (Units)	Protein (mg)	Volume of fraction (ml)	Yield (%)	Specific Activity (U/mg)	Purification Factor (Enrichment)
Crude Extract	5955.6	264.9	28	100	22.5	1
IMAC (Ni- NTA) Fraction	5000	8	20	84	625	27.8
Ultrafiltration 10000 Daltons Cut-off	4902.7	7.5	10	82.3	653.7	29

Table 5: Purification data of His-tagged CpSADH with Ni-NTA superflow matrix.

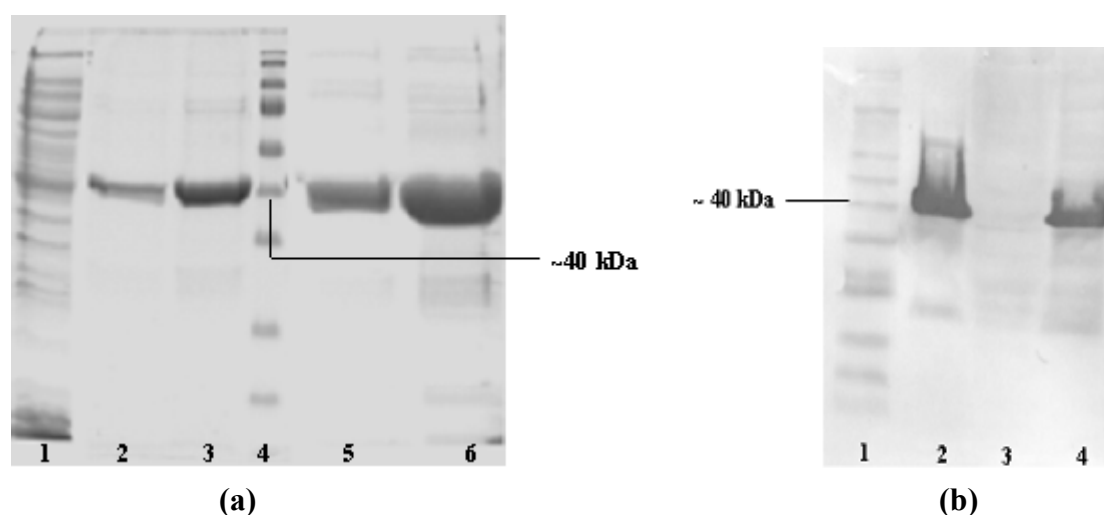


Figure 27:

(a): SDS-PAGE analysis of different fractions of His-tagged CpSADH purified through IMAC using Ni-NTA superflow matrix. Lane 1= Crude extract of CpSADH; Lane 2 = IMAC fraction of His-tagged CpSADH (4 μ l loaded on gel); Lane 3 = Concentrated and desalted purified his-tagged CpSADH after ultrafiltration (4 μ l loaded on gel); Lane 4 = PageRuler™ Prestained Protein Ladder; Lane 5 = IMAC fraction of His-tagged CpSADH (10 μ l loaded on gel); Lane 6 = Concentrated and desalted purified his-tagged CpSADH after ultrafiltration (10 μ l loaded on gel).

(b): Western-blot analysis of the His-tagged CpSADH. Lane 1 = Protein Ladder (Invitrogen); Lane 2 = Concentrated IMAC fraction of CpSADH after ultrafiltration; Lane 3 = Flow-through of the IMAC purification steps; Lane 4 = IMAC purified fraction of CpSADH.

The specific activity of the recombinant His-tagged CpSADH purified by IMAC using Ni-NTA superflow matrix increased from 22.5 U/mg to 625 U/mg and a further up to 653.7 U/mg followed by ultrafiltration. This increase in the specific activity after ultrafiltration and desalting could be due to the removal of imidazole which was de-stabilizing the enzyme (which in turn was showing less activity). A final yield of as much as 82.3% was achieved. The enrichment factor reached 29 fold. All these values are much better than those obtained by purifying CpSADH with anion-exchange chromatography. Using Q-Sepharose FF matrix, Bönitz (2001) reported that the recombinant CPCR reached a specific activity of 32.1 U/mg and a purification factor of 13.9. But the specific activity of the recombinant CPCR as stated by Bönitz reached finally up to 1199 U/mg after purification with 5' AMP-Sepharose 4b. But, the advantage of IMAC purification is that proteins can be purified in one-step, thus saving time, and with high purity enough for industrial applications.

3.2.3 Conclusion of the expression of recombinant CPCR and CpSADH

The expression of recombinant CPCR that had been cloned in this work showed that the protein was expressed in both the *E.coli* hosts JM109 and BL21(DE3) and the specific activities of the expressed protein was 0.4 U/mg and 0.5 U/mg respectively. But the problem in the expression of this recombinant CPCR was that after about 2 rounds of expression, the protein was not expressed anymore and the specific activities were also decreased after every successive expression. Alteration of the expression parameters such as media, growth temperature, IPTG concentration, induction time, did not express the protein. Inclusion bodies were not formed either. Possible reasons could be that the protein was subjected to degradation by proteolytic activities of the bacteria or instability of the N-terminal amino acids. To prevent degradation, protease inhibitor PMSF was added but without success. The functions of many *E.coli* proteases are not known (Makrides, 1996) and it could be that inhibitors like PMSF are not sufficient to prevent such degradation as several *E.coli* proteases are involved in the degradation of a given protein substrate. An alternative is to target the protein to the insoluble fraction of the cell, as inclusion-body proteins are generally protected from degradation. Another possibility that could be used for the expression of the recombinant CPCR was to express it with a fusion protein that stabilizes the N-terminal amino acids. Due to the problems in the expression of the recombinant CPCR that had been cloned in this work, for necessary comparisons, a recombinant CPCR (also based on the known partial amino acids sequences of the wtCPCR) that had been prepared by X-Zyme (Dusseldorf, Germany) was used instead.

The recombinant CpSADH both with and without His₆-tag were successfully expressed and were able to be purified. The CpSADH (without His₆-tag) was purified by anion-exchange chromatography using the Macro Prep High Q matrix. The purified protein had a specific activity of 97.5 U/mg, 10.8 fold enrichment and a yield of 49.8%. But a problem in such purification was that the contaminations with other cell proteins were comparatively higher. Therefore further purification steps would be required.

The recombinant His-tagged CpSADH was purified by IMAC using a Ni-NTA superflow matrix. The purified protein had a specific activity of 653.7 U/mg, an enrichment factor of 29 fold and a final yield of 82.3%. The results showed that the level of purification of the recombinant protein was enough for any industrial applications. Thus the His-tagged CpSADH was purified very easily in just one step with a high purity.

3.3 Characterization of recombinant CpSADH

In order to determine if the His-tagged recombinant CpSADH had undergone any conformational change or modification due the addition of the histidine residues on its N-terminal, the temperature and pH optimum of the IMAC purified protein was compared to that of the CpSADH (without His₆-tag) that had been purified though anion-exchange support. It was found that the temperature and pH optima of both the recombinant CpSADH (with and without His₆-tag) were within the same range. The results shown therefore are for the His-tagged CpSADH as all further experiments were carried out with it due to its higher purity form. For necessary comparisons though, the purified recombinant CpSADH (without His₆-tag) was also used as reference.

3.3.1 Temperature optimum and temperature stability

The activity of the recombinant His-tagged CpSADH was measured at different temperatures ranging from 25 °C to 58 °C for a period of 1 minute using the standard photometric assay for microtiterplates. The temperature optimum is shown in Figure 28.

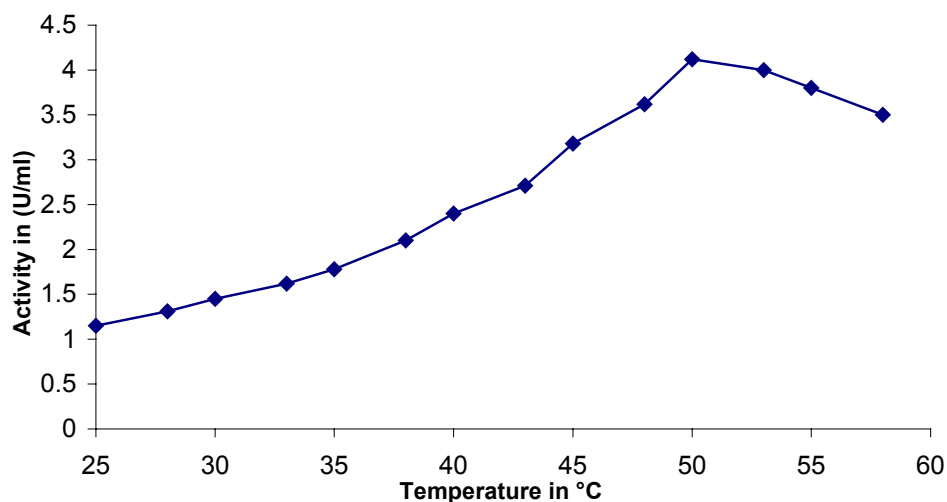


Figure 28: Temperature optimum of the recombinant His-tagged CpSADH photometrically determined for 1 minute.

The maximum activity was found at 50 °C. At 53 °C about 97% and at 55 °C about 92% of the relative activity was measured. The results are in good agreement with those that had been described by Kojima (1995) where the temperature optimum measured for the wtCpSADH was 50 °C. At 55 °C, 88% of the relative activity was measured for the wtCpSADH. The temperature optimum described by Liese (1998) for the wtCPCR was between 50-55 °C. For the wtCPCR, Peters (1993, 1993a) and Bönitz (2001) described the temperature optimum for reduction to be 37 °C and Peters (1993, 1993a) described the temperature optimum for oxidation to be between 50-56 °C. In the work from Steinsiek (2006), the optimum temperature for the wtCPCR was described to be 48 °C which is in good agreement with the temperature optimum of the recombinant CpSADH.

Plotting the natural logarithm of the activities against the reciprocal of the absolute temperatures within the linear range (here between 25 °C and 50 °C), the activation energy of 41 kJ/mol of the recombinant His-tagged CpSADH was determined. This result is in good agreement with Peters (1993, 1993a) where the activation energy of 41.6 kJ/mol was determined for the wtCPCR for the reduction of ketones. Steinsiek (2006) described the activation energy of the wtCPCR to be 43 kJ/mol. The activation energy of the CpSADH was not reported by Kojima (1995).

Temperature dependent half-life of the recombinant His-tagged CpSADH was further determined by measuring the residual activities at regular time intervals, after incubating the enzyme in a solution containing 100 mM TEA-HCl (pH 7.5) at different temperatures ranging from 4 °C to 45 °C for a period of 24 hours. An increase in the reaction temperature not only increased the activity but also decreased the stability of the enzyme. Therefore, temperatures higher than 45 °C were not used. Table 6 shows the half-life and the stability of the enzyme at different temperatures.

Temperature (°C)	Half-life, τ (h)
4	990
21	347
25	289
28	105
30	69
33	39
37	34
40	23
45	18

Table 6: Half-life of the recombinant His-tagged CpSADH.

The highest stability of the enzyme was found at 4 °C with a half-life of approximately 41 days. The enzyme showed very high stability at lower temperatures, but with an increase in temperature, the decrease in the stability was higher/faster. The half-life of the enzyme was reduced with an increase in temperature from 21 to 28 °C about three times. Again from 28 °C to 37 °C the half-life decreased to about three times. From 40 °C onwards, the half-life of the enzyme was below 24 hours. Kojima (1995) described that even after incubation at 40 °C for 10 min, more than 90% of the original activity of wtCpSADH was retained. The period of incubation was too short to determine the half-life. Moreover the half-life of the recombinant CpSADH that had been cloned by the same group was not mentioned. His-tagged recombinant CpSADH had a half-life of 23 hours at 40 °C (more than 99% of the original activity after 15 min) when measured for a period of 24 hours. The stability of the wtCPCR as reported by Steinsiek (2006) was very low when compared. The reported half-life of the wtCPCR at 4 °C was about 18 days but with increase in temperature from 20 to 25 °C, the

half-life decreased rapidly about three times (from 92 h to 32 h). The recombinant His-tagged CpSADH was much more stable at those temperatures (see Table 6). But from 28 °C onwards, the half-life of the recombinant CpSADH was decreasing comparatively faster. From 40 °C onwards the half-life fell to less than 24 hours but comparatively were higher than the wtCPCR as has been reported by Steinsiek (e.g. half-life at 37 °C of only 16.6 h, at 40 °C of only 13.9 h and at 46 °C of only 10.9 h). The half-life of the recombinant CPCR (Bönitz, 2001) and the recombinant CPCR (X-Zyme) are not described.

3.3.2 pH optimum and pH stability

The pH optimum for reduction as well the stability of the recombinant His-tagged CpSADH were determined at different pH ranges using 100 mM TEA-HCl buffer. Figure 29 describes the results of the relative activities that had been determined at different pH values of the buffer and the stability of the CpSADH at different pH values calculated over the residual activity for a period of 24 hours.

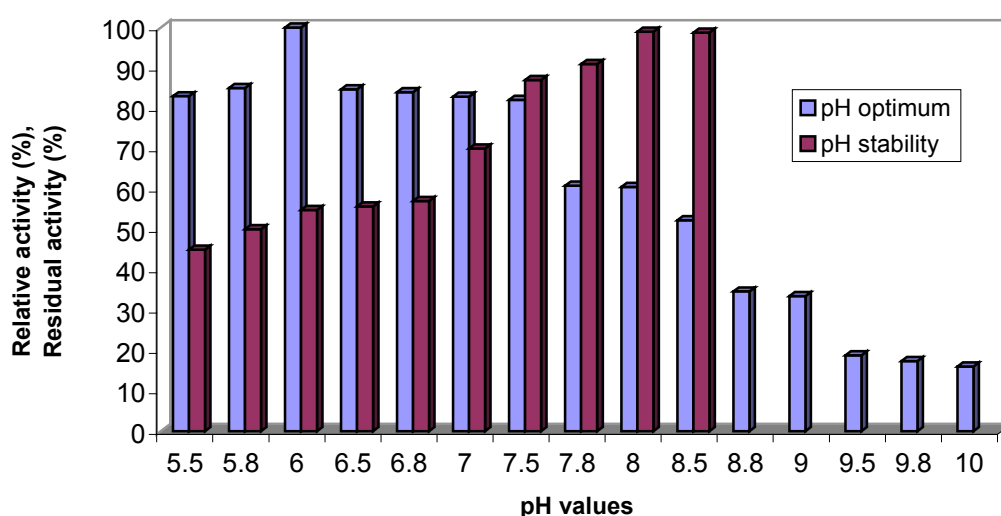


Figure 29: pH optimum and pH stability of the recombinant His-tagged CpSADH.

The highest activity of the enzyme was determined at pH 6. Between pH 5.5 and 6.8 as much as 84% and between pH 7 and pH 7.5 approximately 83% of the relative activity was retained. The relative activity decreased from pH 7.8 (approximately 60%), followed by a further decrease to as much as 34% at pH 8.8. The lowest relative activity of only 16% was measured at pH 10. As the activity was very low from pH 8.8 onwards, the stability of the enzyme in

different pH values was measured only between pH 5.5 and pH 8.5. The highest stability of the enzyme was found within the range of pH 7.5 and pH 8.5. The highest residual activity of 99% was measured at pH 8. A residual activity of 87%, 91% and 98% were measured at pH values of 7.5, 7.8 and 8.5 respectively.

The pH optimum for reduction of the wild type CpSADH was stated to be within the range of pH 5.5- 6.5, the highest being at pH 6 (Kojima et al., 1995). The stability of the same enzyme was stated to be within the range of pH 8 to 10. The pH optimum of the recombinant CpSADH cloned by the same group was not determined. The pH optimum of wtCPCR (Steinsiek, 2006) was likewise determined also at pH 6 and the stability between pH 7.5 to 8, the same as has been determined for the recombinant His-tagged CpSADH. This shows that the recombinant CpSADH and the wtCPCR have very similar pH optimum and also similar pH stabilities.

3.3.3 Stability in hexane

In order to carry out enzymatic synthesis with the recombinant His-tagged CpSADH in aqueous-organic two-phase systems, the stability of the enzyme in the organic solvent (hexane) was very important to know. Many biocatalysts are known to be highly unstable (Gröger et al., 2003, Gröger et al., 2004) and inactivated by organic solvents, either through the solvent or through the interface in between the aqueous and organic phases (Ghatorae et al., 1994; Halling, 1994; Halling et al., 1998). Various groups studied the stabilities of alcohol dehydrogenases in organic solvents. Sensitivity of alcohol dehydrogenases with organic solvents is a very common phenomenon. This has been described for alcohol dehydrogenases like the YADH and the HLADH (Keinan et al., 1986) and also for an alcohol dehydrogenase from *Lactobacillus minor* (Gupta et al., 2004).

The stability of the recombinant CpSADH was studied in different percentages of the organic solvent (hexane). In order to be able to compare the results, the method of determination of the stability was however kept similar to the method described by Steinsiek (2006). The hexane phase consisted of 25%, 50% and 75% (v/v) of the total volume. The aqueous phase that consisted of 2 U of the enzyme dissolved in 100 mM TEA-HCl (pH 7.5) was overlaid with the organic phase in a total volume of 1 ml. The lower phase was mixed with a magnetic stirrer without intermixing the two phases. The stability of the His-tagged CpSADH in contact with organic solvent (hexane) was thereby determined in this aqueous-organic two-phase

system at a temperature of 25 °C. The stability of the enzyme calculated over the half-life in a two-phase system with different percentages of hexane is summarized in the following table.

Percentage of Hexane phase	Half-life, τ (h)
25%	188.4
50%	133
75%	77

Table 7: Half-life of the recombinant His-tagged CpSADH in two-phase system consisting of buffer and hexane at 25 °C.

The half-life of the recombinant His-tagged CpSADH decreased with an increase in the volume of the hexane phase. This could be explained by the fact that the ratio of the interface to the volume of the water phase increased with the rising portion of the hexane phase, since the total volume was kept constant (1 ml in each case). Therefore, with an increase in the volume of the hexane phase, the aqueous phase decreased and thus a more intensive contact between the enzyme and the organic solvent took place than with a larger aqueous phase, in which the enzyme came in contact with the interface more rarely. The recombinant His-tagged CpSADH in 100 mM TEA-HCl (pH 7.5) had a half-life of 289 h at 25 °C without any contact with the organic solvent hexane (see section 3.3.1). In the presence of the organic solvent through the interface, the half-life decreased to 188.4, 133 and 77 hours in 25%, 50% and 75% of hexane phases respectively. That means a stability of approximately 65.2% (with 25% hexane), 46.1% (with 50% hexane) and as low as 26.6% (with 75% hexane) of the original stability that the enzyme had at 25 °C and without any contact with the organic solvent was retained. This showed that the recombinant CpSADH had a high sensitivity against hexane. But the stability of the recombinant CpSADH was much higher than the wtCPCR in hexane under the same conditions. Steinsiek (2006) reported that the half-life of the wtCPCR in hexane was only 45 h (in 25% hexane), 42 h (in 50% hexane) and 22 h (in 75% hexane). Some dehydrogenases are reported to be more stable in hexane than the recombinant CpSADH. Examples are a mutant of the recombinant FDH from *C.biodinii* that was reported to have a residual activity of as much as 92 and 90% in 10 and 20% (v/v) hexane respectively even after 60-69 h (Gröger et al., 2003). But the same enzyme was found to be highly unstable in other organic solvents. Gröger et al. (2003) also found the (*S*)-specific

alcohol dehydrogenase from *Rhodococcus erythropolis* to be stable for long periods under the same conditions (10-20% hexane).

The half-life of the recombinant His-tagged CpSADH after incubation in pure hexane (without any water molecule) was also determined at 25 °C by using a lyophilized powder of the enzyme. It was found that the enzyme under these conditions had a half-life of as low as only 2.2 h. In direct contact with hexane, the enzyme therefore showed very high instability and just after 15 min approximately 90.3% and after 45 min approximately 78.5% of the residual activities was determined. The HLADH showed an exceptional stability in direct contact with pure hexane and as much as 90% of the residual activity was found after 20 hours (Andersson et al., 1998). Steinsiek (2006) reported that the wtCPCR had a residual activity of about 87% after 15 min of incubation in pure hexane. The stability of the cofactor nicotinamide adenine dinucleotide is not affected by organic solvents (Adlercreutz, 1996). Therefore, despite the reduced half-life of the recombinant His-tagged CpSADH in the presence of hexane as organic solvent, the use of this solvent in two-phase system seems to be one of the alternatives to carry out synthesis of hydrophobic substrates, which otherwise are soluble in aqueous medium only partially or in small concentrations and therefore the overall productivity of the reaction medium is decreased.

3.3.4 Inhibition study

The inhibition of the recombinant CpSADH (with and without His₆-tag) when exposed to various reagents was determined to find out their influence on the activity of the enzyme. Of the reagents tried, both the recombinant CpSADH (with and without His₆-tag) were markedly inhibited by ZnCl₂. The same was stated for the CpSADH (Kojima et al., 1995). Peters (1993), Kula et al. (1996), reported that the wtCPCR was strongly inhibited by Zn²⁺ as well. But Bönitz (2001) reported that the CPCR was not inhibited by low concentrations of Zn²⁺, only higher concentration (5 mM) showed some inhibition (72% residual activity). This result of course is different from those that had been stated by Peters, Kula and also Kojima. The recombinant CpSADH showed no inhibition by Fe³⁺ and also not by the chelator EDTA. But the chelator o-phenanthroline inhibited the enzyme. This showed that the enzyme needed an essential metal ion which is removed by o-phenanthroline (hence loss in activity) but not by EDTA (Kula et al., 1996). Kojima et al. (1995), Bönitz (2001) and Peters (1993, 1993a) also described similar results. In the presence of up to 10% DMSO, about 50% of the residual activity of the recombinant CpSADH was retained. Sulfhydryl-protective reagents like DTT

were stabilizing the recombinant CpSADH (both with and without His₆-tag) and as much as 100% of the original activity was retained even after incubation with 1 mM DTT at 30 °C. This result is very much different from what had been reported by Kojima (1995) for the wtCpSADH. Kojima (1995) stated that the wtCpSADH was significantly inhibited by DTT. Peters (1993), Kula (1996) and Bönitz (2001) reported that the wtCPCR was not inhibited by DTT and was similarly stabilizing the enzyme as had been found for the recombinant CpSADH in this research work. Steinsiek (2006) described also that DTT was stabilizing the wtCPCR. The results of the influence of different reagents on the recombinant CpSADH are summarized in the following table.

Reagents	CpSADH			CpSADH (His ₆ -tagged)		
	<u>Residual activity (%)</u>			<u>Residual activity (%)</u>		
	0.5 mM	1 mM	10 mM	0.5 mM	1 mM	10 mM
DTT	95.6	100	n.d.	96	100	n.d.
EDTA	98	98	79	100	100	80
o-Phenanthroline	23	14	n.d.	22	14	n.d.
FeCl ₃	98.7	99	n.d.	99.6	100	n.d.
ZnCl ₂	14.7	0	n.d.	12.9	0	n.d.
DMSO	5%	10%	15%	5%	10%	15%
	59	50	36	58.5	56.9	32

Table 8: Influence of different reagents on the activity of the recombinant CpSADH. n.d. = not determined.

3.3.5 Substrate spectrum of recombinant CpSADH

The substrate spectrum of the recombinant CpSADH (with and without His₆-tag) was measured photometrically. It was found that there were some differences in the reaction conditions (particularly pH of buffers) that were used to determine the substrate spectrum of wtCPCR in the published results. Peters (1993) reported to use a pH of 7.5 (TEA-buffer), Kula et al. (1996) reported to use a pH of 7.8 also in TEA-buffer and Bönitz (2001) reported to use a pH of 7 (TEA-buffer). Other conditions like the concentration of substrate (8 mM) and that of the cofactor NADH (0.2 mM) that had been used in the assays were the same as described by each author. The conditions that had been used to determine the substrate spectrum of the wtCpSADH (Kojima et al., 1995) were not properly mentioned. Besides, the

concentrations of substrates were also not kept constant in each case. According to the data-sheets of JFC, 35 mM of the substrates were used in 100 mM citrate buffer (pH 6.8). But many substrates that are mentioned in the data-sheets are not soluble in water up to that extent. Due to these differences in the published data, the activities of the commercially available purified wtCPCR (Juelich Fine Chemicals, Juelich, Germany) and the recombinant CPCR preparation (X-Zyme, Dusseldorf, Germany) in different substrates were also measured in parallel to the recombinant CpSADH to bring about a direct comparison under the same reaction conditions and also to minimize the errors caused by multiple handling.

The reducing activities of the above-described enzymes are summarized in Table 9 as compared with the standard substrate ABEE reducing activity (100%).

Substrates	CpSADH	CpSADH (His ₆ -tagged)	wtCPCR (JFC)	Recombinant CPCR (X-Zyme)
ABEE	100	100	100	100
Acetophenone	52.1	52.1	48.1	12.3
2'-Chloroacetophenone	8	8.37	--	3.5
3'-Chloroacetophenone	70.7	78.2	63.4	149.8
4'-Chloroacetophenone	88.5	94	97.5	88
2-Butanone	90	94.3	93.3	2
2,4-Pentanedione	58.9	55.6	53.7	3.7
Ethylacetoacetate	91.2	94	88.2	156.6

Table 9: Comparison of the substrate spectrum of different enzymes (reducing activities). The results are expressed as relative activities (in %) as compared with the standard substrate ABEE set as 100%. The reaction mixture contained in a total volume of 1 ml, 100 mM TEA-HCl (pH 7), 8 mM of the substrate, 0.2 mM NADH+H⁺ and 1 unit of enzyme per ml. The activity was measured photometrically at 340 nm and 37 °C for 1 min. (--) = Activity could not be measured.

The pattern of reduction of different substrates with the recombinant CpSADH (with and without His₆-tag) and the commercially available wtCPCR (JFC) were all within the same and very close ranges. It should be noted that the activity with 2'-chloroacetophenone (■) could not be measured photometrically when an enzyme concentration of 1 U/ml was used in the reaction. The reduction of the substrate was initiated only when a high starting concentration of the enzyme was used (25 U/ml for recombinant CpSADH both with and without His₆-tag;

and approximately 60 U/ml for recombinant CPCR preparation by X-Zyme). The concentration of the wtCPCR preparation from Juelich Fine Chemicals (JFC) was only about 17 U/ml (as supplied form). The activity with the substrate was therefore not possible to measure for this enzyme preparation. Literature data of the wtCPCR is reported to have no activity with 2'-chloroacetophenone as well. The probable reason could be the initial start concentration of the enzyme (1 U/ml) that was used (Peters, 1993). Among the chloroacetophenone(s) examined, it was found that the position of the substituent (Cl) in the phenyl-ring possessed an influence on the rate of turnover. The highest reaction rate was reached with the para-substituted chloroacetophenone, followed by the meta-substituted chloroacetophenone. The lowest relative activity was found with the halogen on the ortho-position. Steinsiek (2006) also reported of such increasing patterns of activities for the wtCPCR, though no activity was found for 2'-chloroacetophenone. The relative activities of the recombinant CpSADH and the wtCPCR (JFC) with aliphatic ketones like 2-butanone were approximately 90-94%. With the keto acid ester (diketo-ester) ethylacetoacetate (EAA), approximately 91-94% relative activities were measured for the recombinant CpSADH (both with and without His₆-tag) and about 88% with that of wtCPCR (JFC). With the diketone (2,4-pentanedione), approximately 55-58% relative activity with recombinant CpSADH and about 53% relative activity with wtCPCR (JFC) were determined. From the pattern of behaviour of the recombinant CpSADH and the commercially available wtCPCR (JFC) it could be possible to state that both these enzymes had very similar reaction rates with the examined substrates. In addition, the recombinant CpSADH (with and without His₆-tag) showed similar relative activities with the substrates.

The recombinant CPCR preparation (X-Zyme) as can be seen from the table, had very different reaction rates with the examined substrates. Very low relative activities of only 12.3%, 3.7% and 2% were measured with acetophenone, 2,4-pentanedione and 2-butanone respectively. For measuring the activities with 2,4-pentanedione and 2-butanone an initial concentration of approximately 60 U/ml of the enzyme had to be used. Low activities with these three substrates were also reported in the research work of Müller (Müller, 2004) for the same recombinant CPCR. Further it was reported that this recombinant CPCR preparation required Mg²⁺ for the conversion of 2-butanone. Such dependence was not reported in any of the available literature data for the wtCPCR. For the chloroacetophenone(s), analogous to the pattern of increase in the relative activities according to the position of the halogen as described for the recombinant CpSADH and wtCPCR (JFC) and also as reported by Steinsiek

(2006) for the wtCPCR, it was found that the recombinant CPCR (X-Zyme) had the highest reaction rate of 149.8% with 3'-chloroacetophenone followed by 4'-chloroacetophenone (88%). The enzyme had the maximum relative activity of 156% with EAA.

The oxidation reaction in which an alcohol is converted to the keto-group was also examined using some aromatic and aliphatic alcohols and diols as substrates with the recombinant CpSADH (His₆-tagged). These measurements were basically done to find out if the recombinant CpSADH was able to carry out the back-reaction as well. Some common alcohols that could be used for cofactor regeneration during the enzymatic synthesis were also used as substrates.

The oxidizing activities of the said enzyme are summarized in Table 10 as compared with the (*S*)-2-butanol oxidizing activity (100%). As the product of the actual standard substrate, which is hydroxy-hexane-acid-ethyl-ester (HHEE), is not commercially available, the oxidation of (*S*)-2-butanol was therefore arbitrarily set as 100%.

Substrates	Relative activity (%)
(<i>S</i>)-2-Butanol	100
(<i>S</i>)-1-Phenylethanol	101
(<i>R</i>)-1-Phenylethanol	3
(<i>S</i>)-1,3-Butanediol	20
Ethanol	2
2-Propanol	82

Table 10: Substrates of the oxidation reaction of recombinant His-tagged CpSADH. The reaction mixture contained in a total volume of 1 ml, 50 mM Tris-HCl (pH 9), 50 mM of the substrate, 2.5 mM NAD⁺ and 10 µl of enzyme solution. The activity was measured photometrically at 340 nm and 37 °C for 1 min.

2-propanol is known to be used as cosubstrate for cofactor regeneration. It is described as the cosubstrate for the regeneration reaction of the CPCR (Peters et al., 1993a, b; Zelinski et al., 1999; Laue et al., 2001) and also of the CpSADH (Endo and Koizumi, 2001; Matsuyama et al., 2002). The same was also used for the regeneration of cofactor in the enzymatic synthesis with recombinant His-tagged CpSADH and was found to be converted with good activity. The highest oxidizing activity of the recombinant His-tagged CpSADH was found with (*S*)-1-

phenylethanol (101%) followed with 2-propanol (82%). Similar results were also described by Steinsiek (2006) for the wtCPCR. But in order to reach an efficient regeneration of the cofactor, it is necessary to use a high concentration of the cosubstrate (Steinsiek, 2006) in order to avoid the back reaction from (*S*)-1-phenylethanol to acetophenone because the activity of the His-tagged CpSADH with (*S*)-1-phenylethanol is somewhat higher than 2-propanol.

The wtCpSADH (Kojima et al., 1995) also showed higher activities with (*S*)-1-phenylethanol (about 89% when 50 mM was used) and with 2-propanol (60% when 100 mM was used).

3.3.6 Conclusion of the characterization of recombinant CpSADH

The recombinant CpSADH (mainly the His₆-tagged) was characterized taking five main aspects into consideration: temperature optimum and stability, pH optimum and stability, stability in hexane, inhibition with different reagents and substrate spectrum. It was found that the recombinant His-tagged CpSADH had a temperature optimum for reduction of 50 °C and activation energy of 41 kJ/mol. The enzyme showed very high stability at low temperatures (up to 28 °C). At 25 °C the half-life of the enzyme was determined to be 289 h. This stability reduced to about 65.2% in presence of organic solvent (188.4 h with 25% hexane phase). The pH optimum for the reaction was found to be at pH 6 but the stability of the enzyme was found within the range of pH 7.5-8.5. The same temperature and pH optima were also reported for the wtCPCR (Steinsiek, 2006). Zn²⁺ and o-phenanthroline markedly inhibited the enzyme. But DTT did not inhibit the enzyme (this result is very different from those that had been described by Kojima). Fe³⁺ and EDTA also did not inhibit the enzyme. The enzyme could convert many substrates; examples are aliphatic, aromatic and cyclic ketones. Due to the differences with the literature data of wtCPCR, the commercially available wtCPCR (JFC) and the recombinant CPCR (X-Zyme) were also measured in parallel for direct comparison. Interestingly, the recombinant CpSADH (both with and without His₆-tag) and the wtCPCR (JFC) showed a very similar pattern of conversion with the examined substrates. The recombinant CPCR (X-Zyme) which is based on the derived amino acids sequences by Bönitz (2001) behaved very differently.

Based on the recent data for the characterization of the wtCPCR (Steinsiek, 2006), direct comparison with other closely associated enzymes [wtCPCR, (JFC) and recombinant CPCR, (X-Zyme)] and also certain similarities like the activation energy, inhibition study, with the

old published data for the CPCR (Peters et al., 1993, 1993a), it seems that the recombinant CpSADH and the CPCR (preparations from JFC, from Steinsiek) could be very similar (if not the same) alcohol dehydrogenase, both from *C.parapsilosis*.

3.4 Computer modeling and simulation of CPCR and CpSADH

Experimental conditions that had been examined to characterize the recombinant CpSADH showed that the enzyme was very similar to the recently characterized wtCPCR (Steinsiek, 2006). Moreover, the examined conditions with regard to the substrate spectrum, inhibition study and activation energy that had been covered within this research work also showed that the recombinant CpSADH behaved very similar to that of the wtCPCR but very differently to the recombinant CPCR preparation (X-Zyme)[in case of substrate spectrum]. In order to try and identify the “Real CPCR” and to have a better understanding to which of the two enzymes that are now known (and also proved) to exist in *C.parapsilosis* DSMZ 70125 strain is actually the “Real CPCR”; both these enzymes were modeled. Computer modeling and simulation of the two enzymes were done by Dr. Paolo Braiuca (University of Trieste, Italy), co-operation partner of the working group “Biocatalysis”, RWTH-Aachen, Germany. The modeling was based on the derived amino acids sequences of the two enzymes namely CpSADH (Kojima et al., 1995) and CPCR (see Figure 20 and Figure 10 respectively) using the NAD-dependent alcohol dehydrogenase (EC 1.1.1.1) from *Sulfolobus solfataricus* (Esposito et al., 2002) as the template molecule. According to the model, it was found that though the two enzymes were very different in their amino acids compositions, they had very close similarities in their active sites and therefore an initial comparison of the active sites of the CpSADH and the CPCR was done. Moreover these structures are more conserved among the ADHs. The active sites comparison of the two enzymes according to the model is shown in Figure 30.

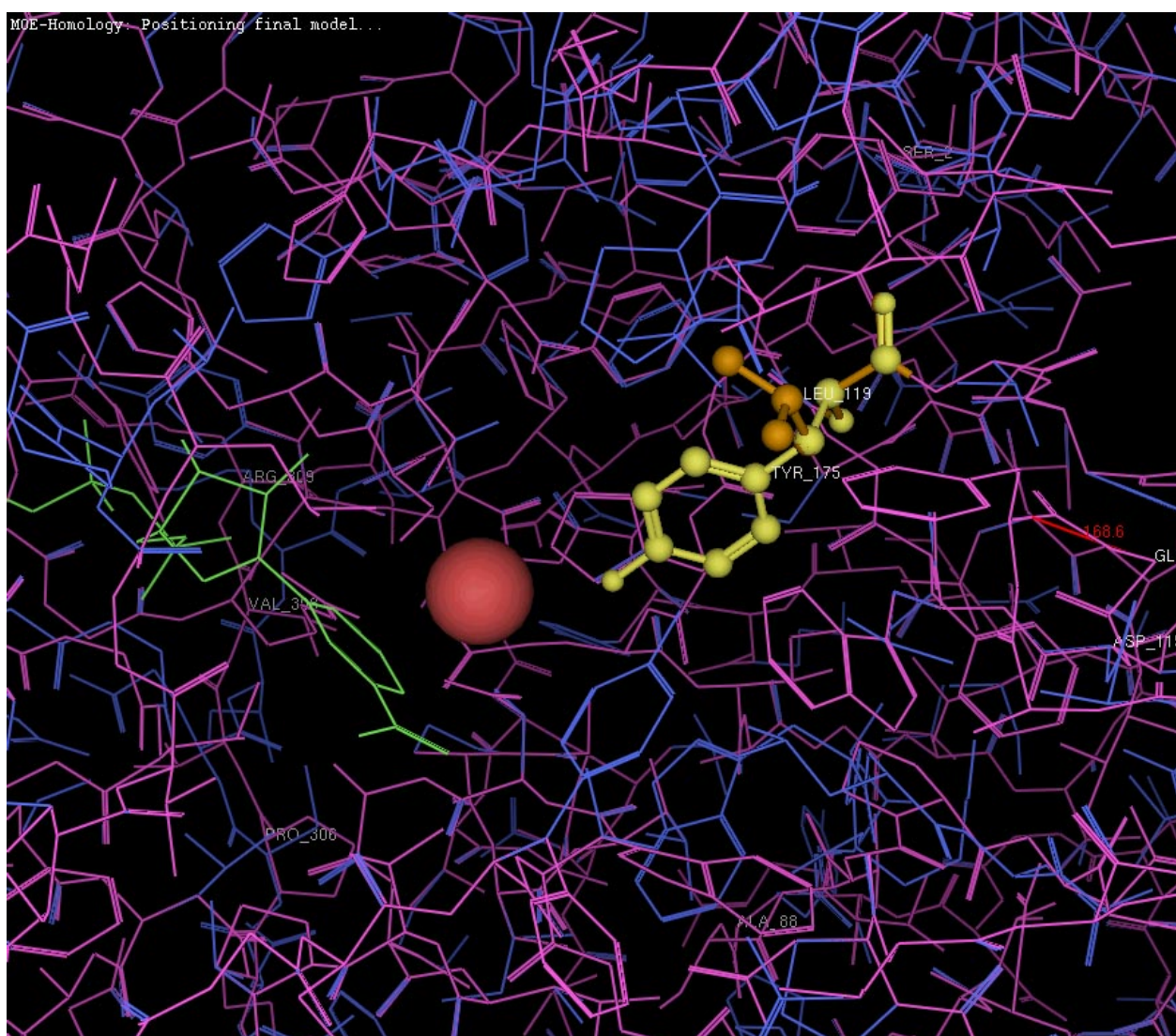


Figure 30: Comparison of the active sites of CpSADH and CPCR.

From the figure it can be seen that the CpSADH has a leucine (LEU) marked in orange at position 119. In contrast, CPCR has a tyrosine (TYR) marked in yellow at position 175 instead. The big red sphere in the center is the zinc ion showing that both the enzymes are zinc-dependent. The green structure on the left side is the NADH molecule. In CPCR, the presence of tyrosine instead of leucine in the active center is an important difference, since the larger tyrosine seems to close a cleft of the active site, which on the contrary is available in CpSADH. It was therefore predicted from the model, that the CpSADH is able to bind somewhat larger substrates.

Another aspect of the model was to examine substrate selectivity of the two enzymes. From the lists of published data available for the broad substrate spectrum (Peters 1993; Peters 1993a; Kula et al., 1996; published data sheets of JFC) that are known to be converted by wtCPCR or tried for conversion with the enzyme, some potential substrates were predicted by the model, which should be converted by the CpSADH and not/very slowly by the CPCR and thus would indicate the identity of the real CPCR. Here the CpSADH is meant by the recombinant enzyme that had been cloned and expressed in this research work using the published amino acids sequences of the same enzyme (Kojima et al., 1995). The CPCR used for comparison was the recombinant CPCR preparation (X-Zyme) which is based on the derived amino acids sequences by Bönitz (2001), the commercially available purified wtCPCR (JFC) and also the wtCPCR preparation by Steinsiek (Steinsiek, 2006). A few of these potential substrates as predicted by the model were used to check for their conversions with the above-mentioned enzymes (recombinant CpSADH, recombinant CPCR preparation by X-Zyme and wtCPCR preparation by JFC and Steinsiek). The conversion rates were measured photometrically at 340 nm. ABEE was used as the standard substrate and was arbitrarily set as 100%. The relative activities hence determined are summarized in the following table.

Substrates	CpSADH without His ₆ -tag	CpSADH with His ₆ -tag	wtCPCR preparation (JFC)	wtCPCR (Steinsiek, 2006)	Recombinant CPCR (X-Zyme)
ABEE	100	100	100	100	100
Methyl-3-oxovalerate	30	27	19	25.2	0
Ethyl benzoylacetate	24.1	23.7	13	28.6	5.6
3-Benzoylpropionic acid	0	0	0	0	0

Table 11: Comparison of some potential substrates as predicted by the model with different enzyme preparations. The results are expressed as relative activities (in %) as compared with the standard substrate ABEE set as 100%. The reaction mixture contained in a total volume of 1 ml, 100 mM TEA-HCl (pH 7), 8 mM of the substrate, 0.2 mM NADH+H⁺ and 10 µl of enzyme per ml. The activity was measured photometrically at 340 nm and 37 °C for 1 min.

As can be seen from the table, the CpSADH and the wtCPCR (both preparations by JFC and by Steinsiek) again showed similar patterns of conversion rates. An important thing to be

pointed out here is that the keto acid ester methyl-3-oxovalerate, which is reported to be converted by wtCPCR even in the known and published literature data (18% relative activity as reported by Peters 1993; Peters 1993a; Kula et al., 1996; JFC data sheets), was not converted by the recombinant CPCR (X-Zyme) which was cloned using the published amino acids sequences (Bönitz, 2001), but was converted by the recombinant CpSADH and also by the wtCPCR preparations. According to the published data, ethyl benzoylacetate that was not converted by wtCPCR, showed conversions with all the enzymes including the CPCR preparation from JFC. This substrate showed the lowest conversion rates with that of the recombinant CPCR (X-Zyme). The keto acid 3-benzoylpropionic acid was not converted by any of the enzymes.

3.4.1 Conclusion of the modeling data

The CpSADH and the CPCR was modeled (courtesy: Dr. Paolo Braiuca) and the active sites of these two enzymes were described in details. It was found that the CpSADH had a leucine residue whereas the CPCR had a comparatively larger tyrosine residue in the active center thus making the former accept more bulky substrates. The model also predicted some potential substrates that should be converted by the CpSADH and not/very slowly converted by CPCR. Accordingly, substrate specificity studies of the recombinant CpSADH, recombinant CPCR (X-Zyme) and wtCPCR preparations (JFC and Steinsiek) showed that a potential substrate methyl-3-oxovalerate was converted by the recombinant CpSADH and also by wtCPCR (JFC and Steinsiek) but not by the recombinant CPCR (X-Zyme). According to the literature data available, methyl-3-oxovalerate was described to be converted by the wtCPCR as well (Peters 1993; Peters 1993a; Kula et al., 1996; JFC data sheets).

These data that were predicted by the model and also verified by experiments confirmed that though both the enzymes were present in the *C.parapsilosis* DSMZ 70125 strain, CpSADH should be the “Real CPCR” with a broader substrate spectrum than the recombinant CPCR (X-Zyme).

3.5 Biocatalytic synthesis in different systems

With the IMAC purified recombinant His-tagged CpSADH, enzymatic syntheses were carried out in different systems. Acetophenone was used as the model substrate. The recombinant

CpSADH as native forms, were used in a pure aqueous system and also in aqueous-organic-two-phase system (the aqueous phase was overlaid with the organic solvent). Furthermore, biocatalytic syntheses with the recombinant CpSADH (His₆-tagged) were also carried out in immobilized forms (section 3.6). Enzymatic substrate-coupled cofactor regeneration was carried out with 2-propanol as cosubstrate.

3.5.1 Enzymatic synthesis in an aqueous system

Substrate-coupled cofactor regeneration

The conversion of acetophenone in a buffered aqueous system (100 mM TEA-HCl, pH 7.5) was carried out with the regeneration of the cofactor NADH+H⁺ by 2-propanol. The solubility of acetophenone, which was used as the substrate, in water/buffer is approximately up to 45 mM. A maximum of this concentration was therefore used for conversions in purely aqueous medium. In order to determine the influence of this cosubstrate, different concentrations of 2-propanol were used within a range between 5 and 20% (v/v). The conversions were approximately between 87.5 to 96.1%. The highest conversion was obtained with 2-propanol concentration of 10% (v/v). The maximum phenylethanol concentration that was formed amounted to 43.2 mM. The results are shown in Figure 31.

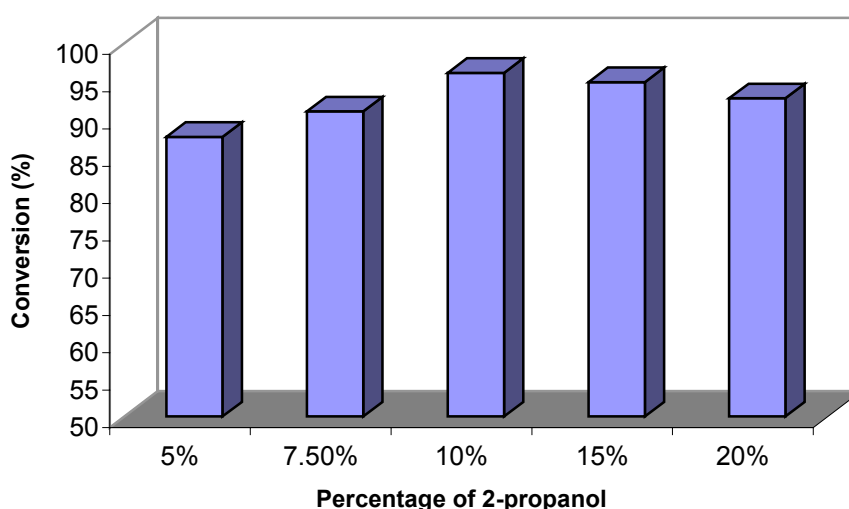


Figure 31: Production of phenylethanol in substrate-coupled reaction with different concentrations of 2-propanol. The reaction was carried out at 30 °C and the reaction mixture consisted of five different concentrations of 2-propanol, 45 mM acetophenone, 1 mM NADH+H⁺ and 0.5 U/ml of recombinant His-tagged CpSADH.

Peters et al. (1993c) described that an optimal substrate conversion by CPCR was achieved with 5% of 2-propanol, whereas Steinsiek (2006) reported that highest substrate conversion by CPCR was achieved with 10% of 2-propanol. As can be seen from the results (Figure 31), the highest conversion with CpSADH was obtained with 10% (v/v) of 2-propanol and thus agreed with the results described by Steinsiek for CPCR. Peters et al. (1993a) reported that the stability of the CPCR was not affected with up to 10% 2-propanol. This was also clearly observed here from the results that had been obtained with the same concentration of 2-propanol. At a concentration of 15% and 20% of 2-propanol approximately 94.8% and 92% conversion was determined respectively. These percentages of conversions were still higher than those obtained with 5% and 7.5% of 2-propanol. The decrease in the conversion rates with 2-propanol concentrations above 10% could be due to the overall decrease of the reductase activity (Steinsiek, 2006) of the recombinant CpSADH, because at such a high concentration of 2-propanol, the back reaction would be favored.

3.5.2 Enzymatic synthesis in a liquid two-phase system

Due to the poor water solubility of many hydrophobic substrates including acetophenone, which was used as the model substrate in the reaction system with recombinant His-tagged CpSADH, the enzyme catalyzed synthesis with acetophenone was carried out in liquid-liquid two-phase system in which the aqueous phase consisted of the enzyme with the cofactor and the organic phase consisted of hexane with the substrate. With this two-phase system a high concentration of the substrate could be used and therefore a better volumetric productivity of the reaction system was achieved. Many alcohol dehydrogenases are described to need a little amount of water in order to be active and be able to convert substrates and just a single water molecule could be sufficient (Zaks and Klibanov, 1988). But usually a higher concentration is needed in order to have a functional two-phase biocatalytic system depending upon the solvent used and/or the enzyme particularly when immobilized by adsorption to the carrier material (Adlercreutz, 1996). In a general way, more the solvent is hydrophobic; higher will be the initial enzymatic activity at low water content (Halling, 1994). Deetz and Rozzell (1988) reported that the minimum quantity of water required for the HLADH and also for the YADH and TBADH is only 0.1% (v/v) in the organic solvent hexane. In the organic solvent n-heptane, the HLADH needed 2.5% of water content in order to exhibit maximum activity (Itozawa and Kise, 1995). The wtCPCR showed activity with just 1% of water content in hexane as organic solvent (Steinsiek, 2006). Depending upon the type of solvent, the activity of the TBADH was between 6 and 30 times better in water than in organic solvent (Jönsson et

al., 1999). In the presence of 0.5% water in isopropyl ether, the activity of the HLADH was approximately one quarter of that in aqueous solution (Zaks and Klibanov, 1988).

If enzyme powder is directly used in organic solvents, its own hydration level could be regarded as a molecular water phase. In order to determine the activity of the recombinant His-tagged CpSADH in hexane with little or no water content, the enzyme (1 U) was lyophilized together with the cofactor NADH+H⁺ for about 20 h in order to be sure that there was no water molecule left and to check if the hydration level after lyophilization was sufficient for catalytic active conformation of the CpSADH (method is described in section 2.4.13). The activity of the enzyme was then examined in four different reaction sets. One of the sets contained no additional water/buffer solution. In the other three sets, buffer was added in a concentration of 0.1, 0.5 and 1% (v/v) of hexane (total volume of the reaction was 1 ml). All the four sets were made in pairs- to one of each pair, 1% (v/v) of 2-propanol was added for the regeneration of the cofactor, while in the other this cosubstrate was not added. The results thus obtained are summarized in Table 12.

Percentage of buffer (%)	(S)-1-Phenylethanol produced (mM)	
	With 1% (v/v) 2-propanol	Without 2-propanol
0	0	0
0.1	2.4	1.9
0.5	15.4	3.3
1	29.2	4

Table 12: Production of (S)-1-phenylethanol in hexane with 0-1% of buffer. The synthesis was carried out at 25 °C using 200 mM acetophenone as substrate in hexane.

It was found that with no additional buffer (both with and without 2-propanol), the CpSADH did not convert the substrate. But, with as low as 0.1% buffer, there was conversion of the substrate and 1.9 mM of (S)-1-phenylethanol was produced. This concentration of the product increased to 2.4 mM when 2-propanol was added for cofactor regeneration. With 1% buffer in hexane, 4 mM (S)-1-phenylethanol was produced and increased further to as much as 29.2 mM when 2-propanol was added for cofactor regeneration. This amount is more than one-half of what had been obtained in a pure aqueous system (43.2 mM phenylethanol), though a lower substrate concentration (45 mM) had to be used due to poor solubility of the substrate

in aqueous solution. It was therefore determined that the lyophilized CpSADH without any water molecule was not active in hexane. But just 0.1% of buffer was enough for the CpSADH to receive a catalytically active conformation and be flexible enough to bind and convert the substrate. For the HLADH it was described that only with addition of very little water or with a water-saturated solvent, the enzyme showed activity when lyophilized together with the cofactor (Grunwald et al., 1986 and Larsson et al., 1991). Some alcohol dehydrogenases are reported to change their stereospecificity by the organic solvents. This is particularly true for the TBADH (Jönsson et al., 1999; Keinan et al., 1984). The recombinant His-tagged CpSADH showed no change in the stereo-selectivity in hexane because only (*S*)-1-phenylethanol was produced.

Further synthesis in aqueous-organic two-phase system with recombinant His-tagged CpSADH was carried out in a larger volume of the aqueous (buffer) phase. The reactions were carried out with 25, 50 and 75% of hexane phase in a total reaction volume of 1 ml. This therefore produced a definite interface. The organic phase consisted of hexane with the substrate while the aqueous phase consisted of the cofactor (NADH, 1mM) together with the enzyme (0.5 U/ml) (see 2.4.13). For cofactor regeneration, 10% (v/v) 2-propanol was used and this concentration was always calculated over the aqueous phase. After 24 hours of synthesis at 25 °C the product formation was analyzed by GC (for the organic phase) and by HPLC (for the aqueous phase). The substrate acetophenone was used in a concentration of 100 mM and 200 mM. This concentration was calculated always over the aqueous-organic phases. Because of its solubility, acetophenone remained mostly in the organic phase, which serves therefore as the substrate reservoir. The substrate diffuses into the aqueous phase, is converted into the product, which is then extracted into the organic phase. After the extraction of the product in the organic phase, the substrate can further diffuse again into the aqueous phase. The concentration of phenylethanol thus produced with the recombinant His-tagged CpSADH in such a two-phase system using 100 and 200 mM of acetophenone as substrate is shown in Figure 32.

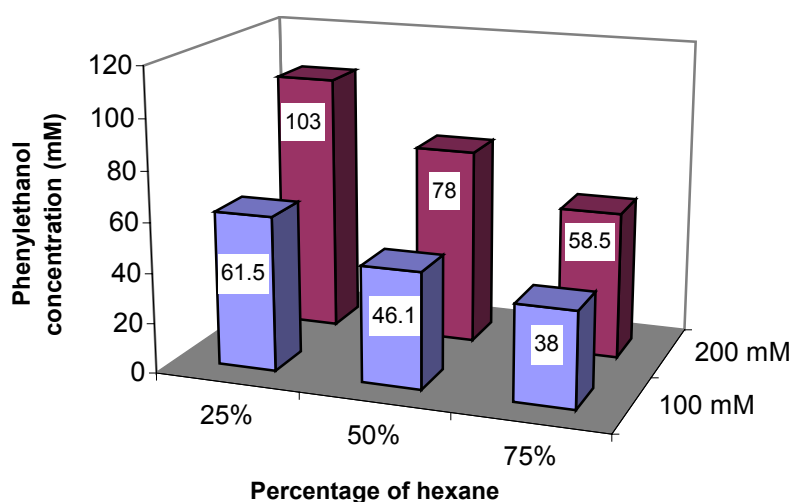


Figure 32: Production of phenylethanol after 24 h with acetophenone concentration of 100 and 200 mM in different volume percent of hexane.

The highest product concentration was found at 25% of hexane phase. 61.5 mM of phenylethanol was produced with 100 mM start-concentration of the substrate whereas as much as 103 mM of phenylethanol was produced with 200 mM start-concentration of the substrate. This yield is relatively better than those that had been described by Steinsiek (2006) for the wtCPCR in which under the same conditions, only 55 mM and 92 mM of phenylethanol was produced (with 100 and 200 mM substrate concentration) after 48 hours. Furthermore, the production of phenylethanol in case of recombinant His-tagged CpSADH was calculated only after 24 hours of synthesis. In the preliminary tests with two-phase system, further conversions after 24 hours of synthesis were not examined though. The lowest product formation took place with 75% hexane phase obviously due to a higher instability of the enzyme, as had been shown earlier, in this percentage of hexane. In comparison to the pure aqueous medium where with the same percentage of 2-propanol for cofactor regeneration, as much as 96% conversions were reached, a worse product formation was determined here. The reason of course could be explained by the inactivating effect of the organic solvent. The larger the organic phase, the greater is the instability of the enzyme. Despite the instability of the enzyme in such a two-phase system, one advantage of such a system is the overall volumetric productivity of the system. In a pure aqueous system, even though about 96% conversion was obtained, only a maximum of 45 mM of acetophenone could be used. Therefore, a maximum of 43.2 mM of phenylethanol was produced. Comparatively, in this two phase system where 100 and 200 mM start-concentrations of the

substrate were used, as much as 61.5 and 103 mM of phenylethanol (with 25% hexane) and, 38 and 58.5 mM of phenylethanol (with 75% hexane) were produced. Product yields in this order of magnitude would not have been possible in a purely aqueous system.

3.5.3 Conclusion of the enzymatic synthesis in different liquid systems

Conversion of acetophenone with the recombinant CpSADH was carried out in different liquid systems. In pure aqueous system, a maximum of 96% (43.2 mM phenylethanol) conversion rate was determined with 10% (v/v) 2-propanol that was used for cofactor regeneration. But one drawback of the pure aqueous system was that, due to the partial solubility of the acetophenone in aqueous solutions, the maximum start-concentration of the substrate (acetophenone) that could be used was only up to 45 mM. This drawback was overcome by using a liquid-liquid two-phase system consisting of hexane and buffer. The hexane served as the reservoir of the substrate and the product could be continuously extracted in the organic phase. Under these conditions, with 25% (v/v) hexane phase, 103 mM phenylethanol was produced with 200 mM start concentration of acetophenone and 61.5 mM of phenylethanol was produced with 100 mM acetophenone. The overall product yields were much higher in comparison to the purely aqueous system. Furthermore, the enzyme showed no activity in the pure organic solvent hexane without any additional buffer, but with just 0.1% (v/v) of buffer the enzyme showed activity and 2.4 mM phenylethanol was produced. With 1% of buffer, as much as 29.2 mM phenylethanol was built.

3.6 Immobilization of recombinant His-tagged CpSADH and enzymatic synthesis with the immobilisate

Immobilization of the enzyme was carried out with two different methods: with entrapment and with covalent binding through adsorption. In the first method, polyvinyl alcohol (PVA) was used because this hydrogels are well characterized and are known to be functional with some alcohol dehydrogenases (Steinsiek, 2006; Metrangolo-Ruiz de Temino et al., 2005). In the second method, ready-to-use enzyme carriers SEPABEADS[®] EC-SERIES (Resindion S.r.l., Italy) were used. The beads were kindly donated by the company for carrying out preliminary experiments with the recombinant CpSADH and check for their functionality with the said enzyme.

3.6.1 Immobilization in PVA and enzymatic synthesis thereof

Immobilization and extensive characterization of the wtCPCR with polyvinyl alcohol beads was successfully accomplished by Steinsiek (2006). Good results and high stability were obtained with an alcohol dehydrogenase from *Lactobacillus kefir* (LKADH) immobilized in the same matrix (Metrangolo-Ruiz de Temino et al., 2005). The entrapment method is useful particularly when an enzyme shows high sensitivity against organic solvents. The immobilized system consists of a gel-stabilized two-phase system, with PVA hydrogel beads and hexane as organic phase. Using this system, the recombinant CpSADH could be used successfully for the reduction of acetophenone.

Enzyme entrapment in PVA was performed by a process of freezing and thawing (Ariga et al., 1987). Gelation of the polyvinyl alcohol solution took place by introducing the solution in drops in cold silicone oil (-70 °C) and then a slow thawing process. During freezing process, phase separation is built through the formation of ice crystals. In the unfrozen low-moisture regions of the system, this remains liquid and the concentration of the polyvinyl alcohol is strongly increased and it forms during the warming-up process, hydrogen bonds between the polymer chains and the free hydroxyl groups. During the slow thawing process the bonds between the PVA chains strengthen. By addition of polyethylene glycol (PEG) and with freezing and thawing, firm hydrogel beads are formed. Complete saponification of the polyvinyl alcohol is required for the stability of the beads (Prüsse, 2000) otherwise the remaining acetate groups would obstruct the formation of hydrogen bonds between the polymer chains (Lozinsky and Plieva, 1998) during gel formation. This saponification was done by addition of sufficient amount of sodium hydroxide in the solution of PVA and PEG prior to gelation (i.e. before adding the solution in silicone oil).

Steinsiek (2006) had done extensive characterization of the wtCPCR immobilized in PVA beads. The recombinant His-tagged CpSADH immobilized in PVA was therefore used for conversion of substrate without further characterization in order to determine the functionality of the immobilisate. In the gel-stabilized two-phase system, consisting of the PVA immobilized biocatalyst and hexane as organic solvent, a fixed ratio of 1:3 of the hydrogel phase to the organic solvent was used such that the immobilisates were completely surrounded by the solvent and thereby a good exchange of substrate and product between the phases was ensured. Due to the high solubility of acetophenone in hexane, the organic solvent served as the substrate reservoir. Enzymatic synthesis was carried out with 200 mM

acetophenone as substrate dissolved in hexane. This start-concentration of the substrate was used because it showed better conversion rates in liquid-liquid aqueous-organic two-phase system. For cofactor regeneration, 2-propanol was used in a concentration of 5 and 10% (v/v), which was calculated over the hydrogel phase. The results are hereby shown in Figure 33.

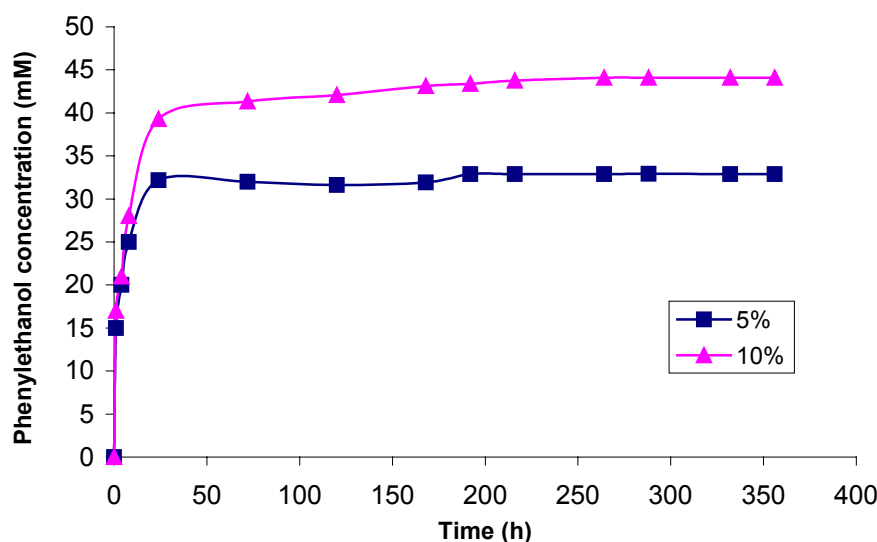


Figure 33: Phenylethanol produced with different cosubstrate concentration. Reaction composition: 2 U/g beads, 1 mM NADH+H⁺, 200 mM acetophenone and 25 °C, pH of the hydrogels were 7.5.

The substrate concentration used amounted to 200 mM, accordingly a conversion of 44.1 mM with 10% (v/v), and of 32.9 mM with 5% (v/v) of 2-propanol was obtained that were detected only with the outgoing concentrations in the organic phase. The difference in the product formation with different 2-propanol concentrations is very much comparable to that of the un-immobilized soluble enzyme in aqueous system. The highest conversion took place with 10% cosubstrate. From the course of the curve, it is evident that there was no inactivation or inhibition of the recombinant CpSADH with the cosubstrate within the examined concentrations.

Apart from the activities of the immobilisate that was determined, there are some other parameters that play a role during the substrate acceptance and product formation. The mass transfer is particularly important since the substrate must first diffuse into the beads and after conversion, the product must diffuse out. The exact concentrations in the beads are not detectable thereby. But by the solubility of the product and substrate in the gel phase, it can be measured how much will remain in it. According to the distribution of the product between

gel and solvent phase, it can be estimated, in which concentrations the product/substrate will remain in the beads. The distribution of phenylethanol between aqueous phase i.e. the hydrogel phase (PVA beads) and organic phase is shown in Figure 34.

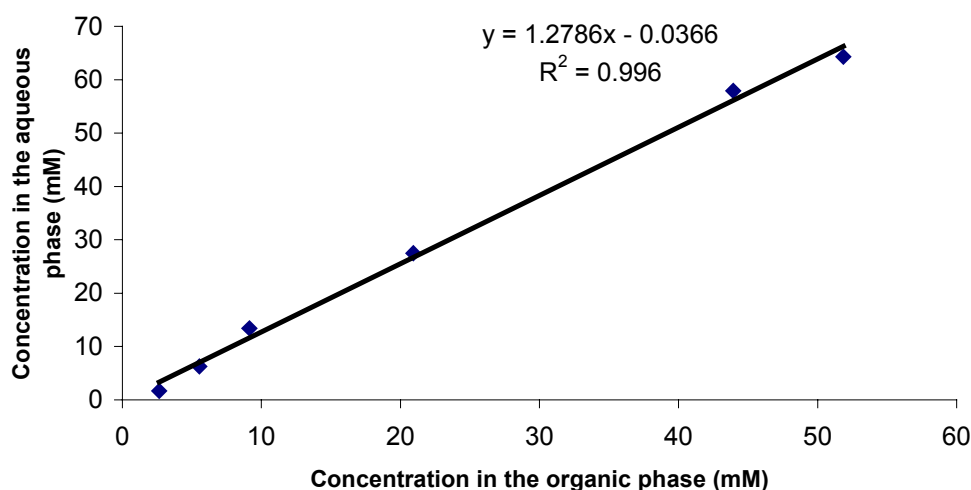


Figure 34: Balanced distribution of phenylethanol between the aqueous and organic phase, determined with a volume ratio of 1:3 (hydrogel phase: organic solvent hexane).

In the examined concentration range (between 2.5 to 52 mM), the concentration of phenylethanol was approximately 1.3 times more in the hydrogel phase than in the hexane phase. When determining the production of phenylethanol with the enzyme immobilized in PVA beads, this distribution was not considered, since the yield was referred to which is actually available.

3.6.2 Immobilization in Sepabeads and enzymatic synthesis thereof

The activity and functionality of the recombinant His-tagged CpSADH were determined in two different types of ready-to-use enzyme carriers SEPABEADS® EC-SERIES namely EC-EP and EC-HFA. Brief characteristics of these two types of beads are described in the following table (source of information: Resindion S.r.l., Italy).

Characteristics	SEPABEADS [®] EC-EP	SEPABEADS [®] EC-HFA
Matrix	Polymethacrylate	Polymethacrylate
Active Group	Epoxide	Amino Epoxide
Functional Group density	Minimum 110 $\mu\text{mol/g}$ wet	Minimum 50 $\mu\text{mol/g}$ wet
Particle Size range	150-300 μm	150-300 μm
Pore Diameter (for target enzyme size: normal)	30-40 nm	30-40 nm
Porosity	> 25%	> 25%
Water Retention	55-65%	50-60%

Table 13: General characteristics of the EC-EP and EC-HFA beads.

EC-EP and EC-HFA perform covalent fixation by a nucleophilic attack of the amino groups of the enzyme on the epoxy groups of the carrier. EC-EP is unpolar and will not adsorb typical enzymes in the absence of high buffer concentration. EC-HFA is more polar allowing enzymes also to adsorb, but later they are immobilized via the epoxy groups. In the EC-EP, first a very slow intermolecular covalent immobilization takes place between the enzymes. The enzyme thereby is bound to the carrier by mild physical adsorption through hydrophobic adsorption plus very fast intramolecular covalent binding (Mateo et al., 2000a; Mateo et al., 2002). In the EC-HFA, a high density of amino-epoxy groups is present. Due to this, the enzymes are adsorbed into the carriers through fast physical adsorption. After the physical adsorption, a very strong covalent immobilization takes place between the epoxy-amine groups (Mateo et al., 2003).

Immobilization with the EC-EP and EC-HFA enzyme carriers

The recombinant His-tagged CpSADH was successfully immobilized on the carriers with the methods described earlier (see section 2.5.2). Since the immobilization method with EC-EP needed a high concentration of buffer, a concentration between 100, 200, 500 and 1000 mM was used to examine the optimal buffer conditions for the immobilization of the enzyme. For the immobilization with EC-EP (and also EC-HFA), potassium phosphate buffer instead of TEA-buffer was used during the immobilization methods and also during syntheses. This was

because, triethanolamine (TEA) has a free electron pair, which could perform a nucleophilic attack on the epoxy group and thereby reduce the overall immobilization yield with the enzyme. It was found that the adsorption of the enzyme on the Sepabeads (EC-EP) was about 80% during the first two hours of incubation with 100 mM phosphate buffer, pH 8. With the other three concentrations of the buffer (200, 500 and 1000 mM) at pH 8 that had been used, the adsorption efficiency was the same (> 99% of bound activity) during the first 2 hours. But, increase in the concentration of the phosphate buffer resulted in a terrible decrease in the stability and activity of the soluble enzyme. This might be owing to the conformational changes in the enzyme caused by the substantial increase in ionic strength leading to loss of enzyme activity. Similar effects of decrease in diastase activity with increasing salt concentrations were observed by Bajpai and Sachdeva (2002). When compared between 200-1000 mM phosphate buffer, the activity and stability of the enzyme was found to be the highest at 200 mM (pH 8) of the phosphate buffer. This concentration was therefore used for the immobilization method since the immobilization yield was the same when compared with the other two concentrations (500 and 1000 mM) of the phosphate buffer. The effect of increasing ionic strengths of the phosphate buffer on the stability of soluble CpSADH and immobilization efficiency on the EC-EP Sepabeads is shown in Figure 35. It was found that at 100 mM phosphate buffer concentration, about 99% of residual activity was retained. This decreased to about 3% at 200 mM buffer concentration. At 1 M buffer concentration, the stability of CpSADH was highly affected and only 56% residual activity was determined.

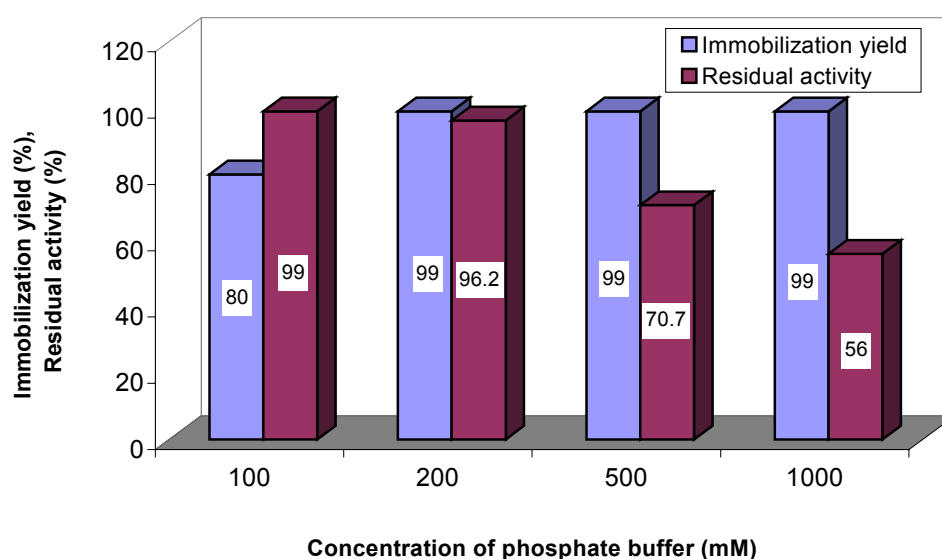


Figure 35: Effect of concentrations of phosphate buffer on the stability of CpSADH and immobilization efficiency on EC-EP Sepabeads.

Due to the high polarity of the EC-HFA, no buffering is usually required. But, the recombinant CpSADH needed a small buffering in order to be stable for a longer time. A very low concentration of 20 mM of phosphate buffer, pH 7.6 was used for the immobilization, which showed to be enough for a long-term stability of the enzyme.

With the optimized conditions for immobilization, it was found that the recombinant CpSADH was completely adsorbed in the EC-HFA Sepabeads within the first 1 hour at 25 °C (> 99% bound activity). The adsorption of the enzyme in the EC-EP was a bit slower; it was only after the first 2 hours of incubation that > 99% bound activity was found. After the adsorption was completed, further incubation was done at 5 °C (in order to prevent thermal degradation of the enzyme) for 48 h by stirring in an over-head shaker. This second incubation was done in order to facilitate covalent binding of the adsorbed enzyme to the Sepabeads. After the immobilization was completed, the supernatant was eliminated after checking the residual activity. The course of immobilization with the EC-EP and EC-HFA Sepabeads are shown in Figure 36.

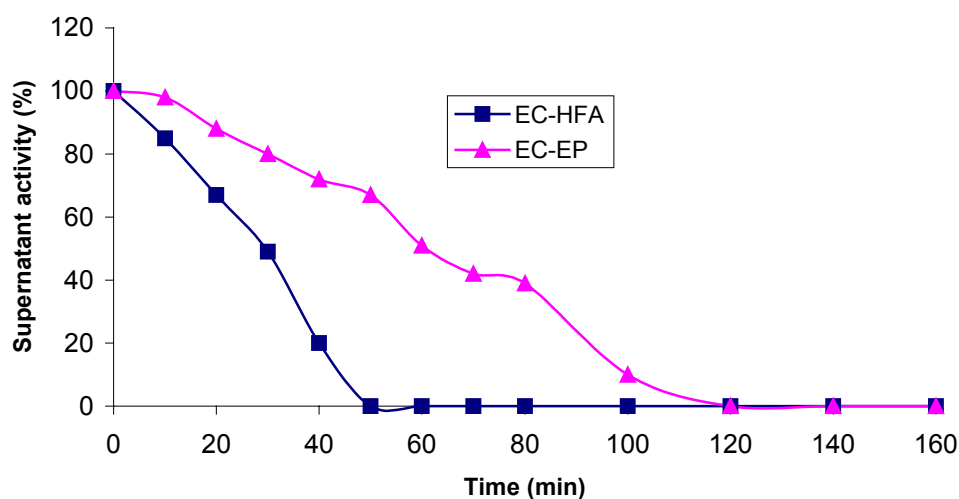


Figure 36: Course of immobilization of the recombinant His-tagged CpSADH on EC-EP and EC-HFA Sepabeads during the first 2 hours. Protein/enzyme loading: 10 units of the enzyme (0.5 mg protein) per gram beads.

From the course of the curve, it is evident that the immobilization was approximately two times faster on the EC-HFA than on the EC-EP. This was checked particularly during the first two hours of immobilization so as to determine the adsorption efficiency of the two kinds of Sepabeads. After the initial two hours when adsorption was completed ($> 99\%$ bound activity determined), a second incubation for 48 h was done to ensure covalent binding as because the process of covalent binding is comparatively slower than that of adsorption. No desorption of the enzyme was found after the immobilization was completed when using 200 mM phosphate buffer at pH 8 for the EC-EP and 20 mM phosphate buffer at pH 7.6 for the EC-HFA and with protein loading of 0.5 mg protein/gram beads. A comparatively rapid immobilization rate particularly during the first two hours with the EC-HFA Sepabeads could be explained by the fact that in the epoxy-amino Sepabeads, the presence of amino groups allows a very rapid ionic adsorption of the proteins onto the support at low ionic strength (here 20 mM phosphate buffer, pH 7.6). On the other hand, using epoxy Sepabeads (EC-EP), enzyme adsorption requires high ionic strength (here 200 mM phosphate buffer, pH 8) and proceeds via the hydrophobic interaction between the external hydrophobic pockets of the enzyme and the hydrophobic surface of the support, which yielded a lower immobilization yield during the first two hours. This greater binding capacity and higher immobilization rates with the EC-HFA Sepabeads were also reported by Mateo et al. (2003) and Torres et al. (2003) for several examined enzymes.

Protein loading

To determine the carrier capacity, increasing amounts of protein was loaded within a range of 0.0001 to 0.001 mg/mg beads. The ratio of the beads to the enzyme/protein solution for immobilization was always kept constant (1:4). A saturation of the surface of the beads with the protein was not found within the studied range and an adsorption rate of $> 99\%$ was accomplished within the first 1 hour of immobilization (with EC-HFA) and within the first 2 hours (with EC-EP) under the optimized immobilization conditions. This showed that the examined range of protein was obviously not the maximum capacity of the carriers. It might have been possible to load with higher amounts of protein, but the preliminary synthetic experiments with the immobilized enzyme were carried out only with 10 units of the enzyme (0.5 mg protein) per gram beads. Synthetic reactions were carried out in a total volume of 4 ml. For such small-scale synthesis (in 4 ml reaction volume), a higher amount of enzyme/protein loading was not required. This low protein load was also chosen to prevent

diffusion problems. No desorption of enzyme from the beads was found when loaded up to 0.001 mg protein/mg beads. This was verified by washing the immobilized beads with buffer very thoroughly and afterwards checking the activity as well as the amount of protein in the solution. Therefore the total immobilization yield obtained was > 99% in each case.

Synthesis with the enzyme immobilized on Sepabeads in pure aqueous system

Unlike the PVA beads, it was not possible to co-immobilize the cofactor NADH+H⁺ along with the enzyme in the Sepabeads. The NADH has to reach the active center, which will be only possible with free or homogenously enlarged cofactor e.g. PEG-NADH. To check the functionality of the enzyme immobilized on Sepabeads, initially all syntheses were carried out in pure aqueous medium (50 mM potassium phosphate buffer, pH 7.6) and NADH+H⁺ was supplied into the bulk phase so as to rule out the possibility of cofactor limitations. The compositions of the reaction mixtures for both the EC-EP and EC-HFA Sepabeads had been described in section 2.5.2 (Reaction set-up for aqueous system). The ratio of the immobilized biocatalyst to that of the reaction volume was always kept at 1:16 (250 mg sepabeads in 4 ml of total reaction volume). Acetophenone was used here also as the model substrate in order to be able to compare different reaction systems. Since the solubility of acetophenone in aqueous medium is only up to 45 mM, therefore this concentration was also used for the synthesis. Cofactor regeneration was done with 10% (v/v) of 2-propanol because the highest activity of the recombinant His-tagged CpSADH was always found at this concentration of the cosubstrate. The phenylethanol that had been produced by the enzyme immobilized on EC-EP and EC-HFA are shown in Table 14.

Time (h)	Phenylethanol produced (mM)	
	With EC-EP	With EC-HFA
24	3.4	34.3
48	5.1	39.4

Table 14: Production of phenylethanol with enzyme immobilized on Sepabeads in pure aqueous medium.

The highest conversion was found with EC-HFA Sepabeads. Since 45 mM acetophenone was used as substrate as much as 87.5% conversion (39.4 mM phenylethanol) was achieved. In a pure aqueous system with soluble enzyme, the percentage of conversion was about 96%. But, a remarkably very low conversion of only 5.1 mM phenylethanol (11.2%) was obtained with the epoxy-linked EC-EP Sepabeads. Additionally, it was found that the material balance (concentration of substrate and/or product) that had been analyzed with the HPLC was very different. It was presumed that after immobilization some of the epoxy groups might remain unconverted and the substrate and/or product when used showed some affinity towards them. Loading with as much as 30 mg protein/gram beads did not change the difference. This material imbalance was true also in the case of EC-HFA but the difference was very less when compared to the EC-EP.

In order to determine how much have the substrate and/or product bound to each of the two kinds of Sepabeads, enzyme-free beads were used and after equilibration was reached (48 hours), the adsorption of the substrate and/or product into the beads was determined. The ratio of beads to reaction volume was always kept at 1:16 (250 mg enzyme-free beads in 4 ml reaction volume). The results are shown in Figure 37 (a-d).

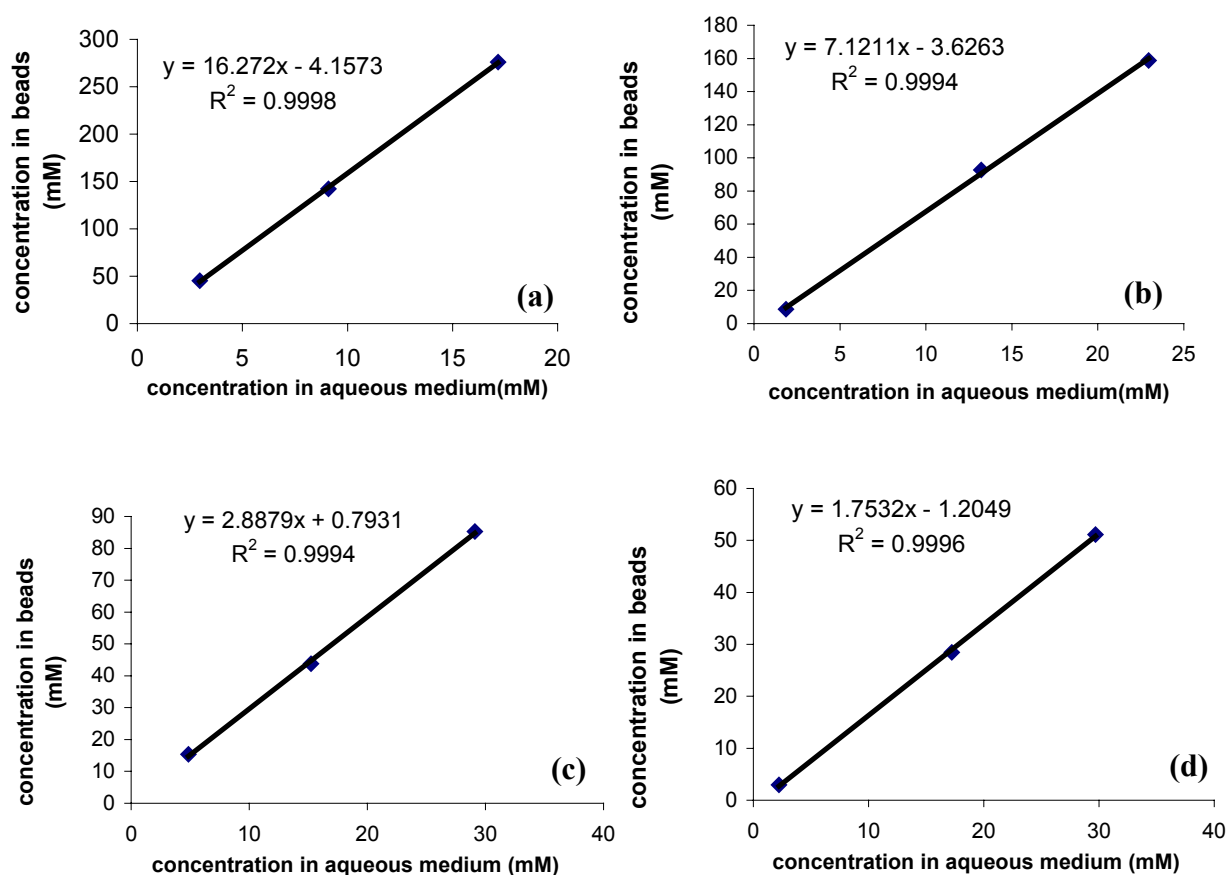


Figure 37: Balanced distribution of acetophenone and phenylethanol between the aqueous medium and the Sepabeads, determined with a volume ratio of 1:16 (Sepabeads: aqueous medium).

- (a): Distribution of acetophenone in EC-EP Sepabeads.
- (b): Distribution of phenylethanol in EC-EP Sepabeads.
- (c): Distribution of acetophenone in EC-HFA Sepabeads.
- (d): Distribution of phenylethanol in EC-HFA Sepabeads.

In the examined concentration ranges, it was found that for EC-EP, the concentrations of acetophenone bound to the beads was as much as 16 times more and that of phenylethanol was as much as 7 times more than in the reaction medium. For EC-HFA, the concentration of acetophenone bound was 2.8 times more and that of phenylethanol was 1.7 times more than in the reaction medium. A comparatively very high affinity of acetophenone towards the EC-EP Sepabeads could be because of its unpolar characteristics. As acetophenone is very hydrophobic, therefore it had more affinity towards the beads than towards the buffer.

Comparatively, phenylethanol is more hydrophilic. Therefore it had a lower affinity towards the EC-EP Sepabeads. On the other hand, EC-HFA Sepabeads is more polar; therefore acetophenone and phenylethanol had lower affinities towards it. Due to this huge material imbalance with the EC-EP Sepabeads, and also very low enzymatic conversions, this was obviously not the right enzyme carrier particularly when carrying out enzymatic synthesis in pure aqueous system.

Synthesis with the enzyme immobilized on Sepabeads in aqueous-organic two-phase system

The functionality of the enzyme immobilized on Sepabeads was examined in an aqueous-organic two-phase system in order to have the possibility of using a higher starting concentration of the substrate acetophenone and thereby have better yields at the end of the synthesis. A small aqueous phase had to be included in the synthetic reactions to supply the cofactor $\text{NADH}+\text{H}^+$. This aqueous phase varied from 0.5 ml and 1 ml in a total reaction volume of 4 ml (the remaining volume consisted of hexane with the substrate acetophenone). Accordingly, two sets of reaction probes were established: SET A and SET B (see section 2.5.2). SET A consisted of 1 ml aqueous phase containing 1 mM $\text{NADH}+\text{H}^+$ in 50 mM phosphate buffer, pH 7.6 and 3 ml of hexane phase containing 200 mM acetophenone (concentration was calculated for the aqueous-organic phases). SET B consisted of 0.5 ml aqueous phase containing 1 mM $\text{NADH}+\text{H}^+$ in 50 mM phosphate buffer pH 7.6 and 3.5 ml of the hexane phase with 200 mM substrate acetophenone (concentration calculated for aqueous-organic phases). Cofactor regeneration was carried out with 10% (v/v) of 2-propanol (concentration was calculated for the aqueous phase only). The ratio of the enzyme immobilized on Sepabeads to the total reaction volume (including the aqueous-organic phases) was kept constantly at 1:16 (250 mg Sepabeads in 4 ml of total reaction volume). Synthesis was carried out at 25 °C and proper mixing of the reaction system was ensured by shaking in an end-to-end shaker. When using Sepabeads, it is very important to take care of the stirring method for proper mixing of the reaction system. Sepabeads being very fine particles, care should be specially taken not to use magnetic stirrers, as these would cause grinding of the Sepabeads. If magnetic stirrers cannot be avoided, triangular sticks or sticks with wheels at its ends could be used as these would not cause grinding of the beads. In any case, round sticks should never be used. The substrate consumption and product formation

were analyzed at regular time intervals via HPLC (aqueous phase) and GC (organic phase). The results obtained thereby are shown in Figure 38 (a-b).

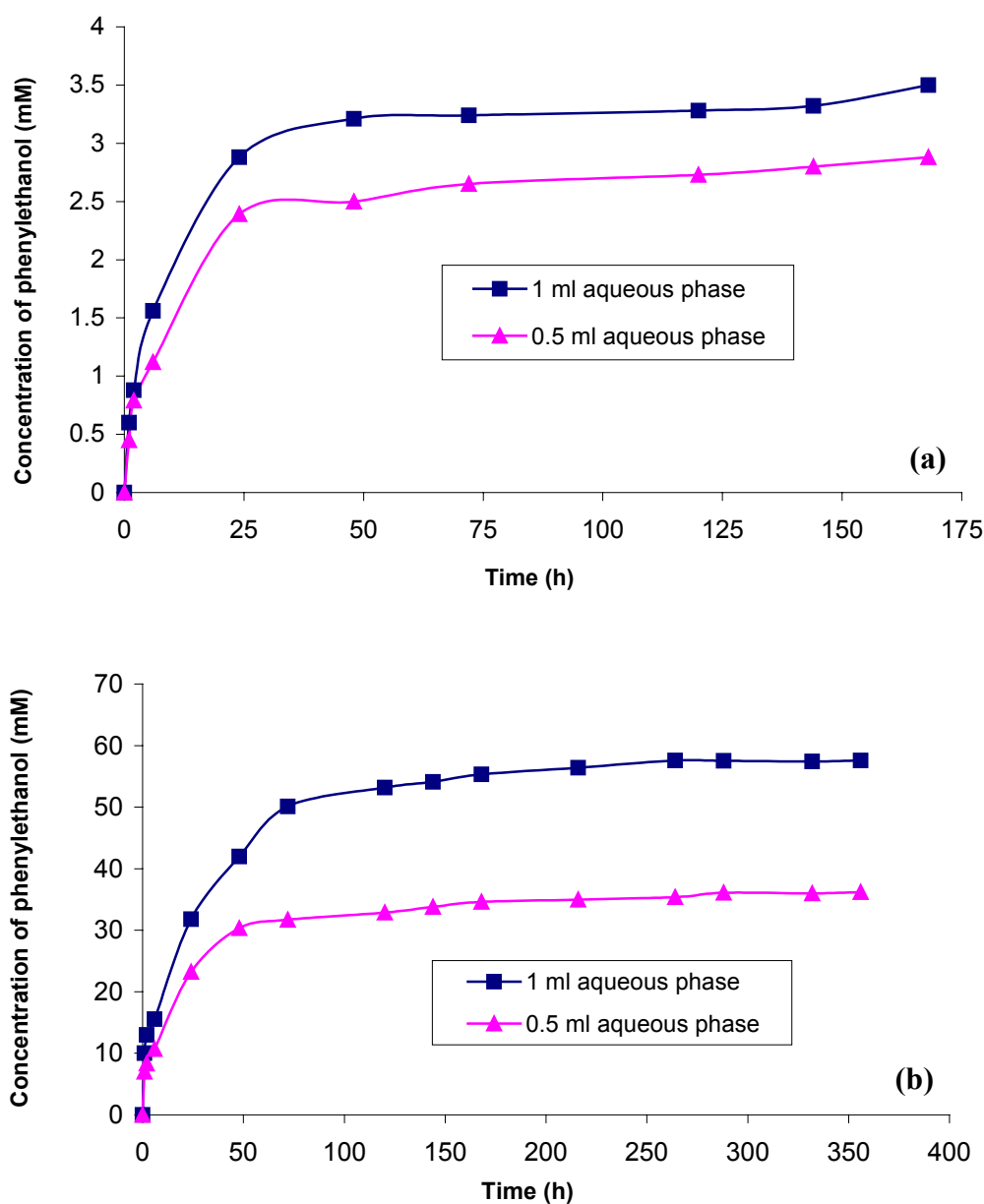


Figure 38: Production of phenylethanol with enzyme immobilized on Sepabeads in a varied aqueous-organic two-phase system.

(a): Enzymatic synthesis with EC-EP Sepabeads.

(b): Enzymatic synthesis with EC-HFA Sepabeads.

In the aqueous-organic two-phase system, the highest conversion was found with the EC-HFA Sepabeads as had been found also in the pure aqueous system. But, among the two reaction sets that had been studied, it was found that the conversion was better when an

aqueous phase of 1 ml to 3 ml hexane phase was used rather than 0.5 ml to 3.5 ml (aqueous phase to organic phase). With the first instance (SET A composition) consisting of 1 ml aqueous phase, as much as 57.6 mM phenylethanol (28.8% conversion) was formed from 200 mM acetophenone. With the second instance (SET B composition) 36.2 mM phenylethanol (18.1% conversion) was built from 200 mM acetophenone. The concentration of the product that was bound in the Sepabeads was not taken into consideration since they were not extracted in the said experiments. The reason behind a comparatively lower conversion rate with the SET B was obviously due to the inactivation/instability of the enzyme in close contact with the organic solvent hexane. Since, in this composition, a ratio of 1:7 in a total reaction volume of 4 ml (1 part aqueous medium to 7 parts organic medium) was used, the contact of the enzyme immobilized on Sepabeads with hexane was more intensive due to an increased ratio of the interface and the aqueous phase. This resulted in a faster instability/inactivation of the enzyme. Comparatively, in the SET A composition, a larger aqueous phase was used (1:3 aqueous to organic phase in a total reaction volume of 4 ml). Therefore, the contact of the enzyme immobilized on Sepabeads with hexane was less intensive, and hence the enzyme was more stable/active, which in turn resulted in a better conversion. With the gel-stabilized two-phase system (enzyme immobilized in PVA beads and hexane in 1:3 ratio) after 356 h, a maximum of 132 μ mol phenylethanol (44.1 mM phenylethanol in 3 ml hexane) was produced that was detected only with the outgoing concentrations in the organic phase and therefore was considered to be the final yield achieved at the end of the synthesis. In contrast, with the enzyme immobilized on EC-HFA Sepabeads in an aqueous-organic two-phase system (in 1:3 ratio of aqueous to organic phase) after 356 h, as much as 230 μ mol phenylethanol (57.6 mM phenylethanol in 4 ml reaction volume) was produced. This value was also calculated taking into consideration only the available amounts in the aqueous and organic phases and hence was the final yield. The concentration of phenylethanol bound to the Sepabeads was not considered. Under these conditions, approximately 100 μ mol more of phenylethanol was produced with the enzyme immobilized on Sepabeads than with the enzyme immobilized in PVA beads.

With the EC-EP Sepabeads, conversion was very less in the aqueous-organic two-phase system. However the conversion was similar to that of the pure aqueous system. The substrate concentration used amounted to 200 mM, accordingly a conversion of only 3.5 mM phenylethanol with SET A composition and 2.5 mM phenylethanol with SET B composition was obtained. The material balance with the EC-EP Sepabeads in aqueous-organic two-phase

system was again studied (beads to reaction volume ratio was 1:16). It was found that for phenylethanol, similar concentrations were bound to the Sepabeads like those that had been found in the pure aqueous system (about 7 times more than in the reaction medium). With acetophenone it was found that when a starting concentration of 200 mM was used, after equilibrium, about 185 mM was staying in the solution of hexane and buffer and about 15 mM was staying in the beads. Therefore enough of the substrate was still left in the solution to be converted by the enzyme. But the enzyme was simply not active/stable enough in the EC-EP Sepabeads in order to carry out a good conversion reaction. This different effect on the enzyme activity of the immobilization when using different supports (even though both of them have epoxy groups as reactive group) may be related to different orientations of the enzyme on the support. This implies that the enzyme likely reacts with the support via different areas (the areas of the protein surface that have more hydrophobic residues or the areas of the protein that have more negative charge) with co-occurrence of different effects on enzyme activity. Moreover, the different nature of the support (hydrophobic or ionic) may also have some relevance to explain the difference in the enzyme activities. A negative effect (less activity) of the hydrophobicity of the support could be because the close presence of a hydrophobic surface (of the enzyme) near the support can promote some unstabilizing effect, similarly like the presence of any other hydrophobic interfaces like organic solvents phase (Mateo et al., 2002).

Stability of the enzyme immobilized on Sepabeads

After determining the functionality with regard to activity of the enzyme immobilized on Sepabeads, it was interesting to know the stability of the immobilized enzyme. Due to the poor conversion rates that had been achieved with the EC-EP Sepabeads, these were not used in the further experiments. But the immobilized EC-HFA Sepabeads showed a very good conversion rate and was even better when compared to the conversion obtained with the PVA beads. The stability (thermo-stability) of the recombinant His-tagged CpSADH immobilized on EC-HFA Sepabeads was therefore necessary to know. Figure 39 shows the residual activities that had been measured with the immobilized enzyme after incubation at different temperatures in an aqueous-organic two-phase system (method described in section 2.5.3). The ratio of the immobilized enzyme to the total aqueous-organic volume was kept at 1:16 [250 mg beads to 4 ml of reaction volume; buffer (1 part; 50 mM phosphate buffer, pH 7.6) and hexane (3 parts)].

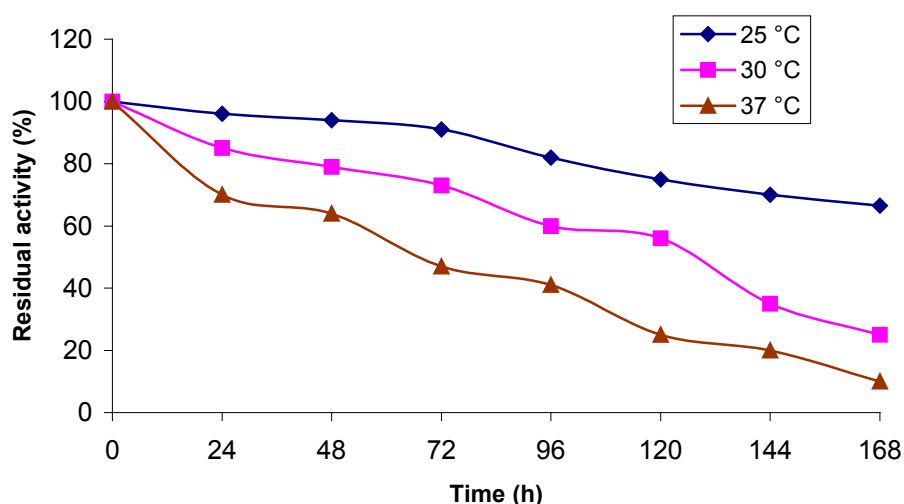


Figure 39: Thermo-stability of the recombinant His-tagged CpSADH immobilized on EC-HFA Sepabeads. Residual activity was measured after storage of the immobilisate at different temperatures.

The half-life of the immobilized enzyme in EC-HFA decreased with an increase in the temperature. At the lowest temperature that had been studied here, the immobilized enzyme had a half-life of approximately 284 h. At 30 °C the half-life reduced to 91.2 h followed by a further decrease to 55 h at 37 °C. In Table 15, a comparison of the half-life of the soluble enzyme (in one phase and two-phase) to that of the immobilized one is shown.

T (°C)	τ (h), soluble enzyme in pure aqueous medium	τ (h), immobilisate (1:3 phase ratio)	τ (h), soluble enzyme in two-phase system (1:3 phase ratio)
25	289	284	77
30	68.6	91.2	Not determined
37	34.2	55	Not determined

Table 15: Comparison between the half-lives of the soluble and the immobilized CpSADH.

At lower temperature (here 25 °C), the half-life of the immobilized CpSADH on the EC-HFA Sepabeads was more or less the same as in the soluble form in one phase. But at higher temperatures, the stability of the immobilized enzyme increased significantly. The half-life of the soluble CpSADH in aqueous-organic two-phase system was comparatively very less (see 3.3.3). It was found that under the same phase ratio of buffer to hexane (1:3), the soluble enzyme had a half-life of only 77 h at 25 °C. In a two-phase system, the half-life of the

immobilized enzyme on Sepabeads therefore increased to approximately 3.6 times when compared to that of the soluble enzyme in a similar two-phase system consisting of the same phase ratios (Table 15). Similar increase in the stabilities of the amino-epoxy immobilized enzymes was also reported by Mateo et al. (2003).

3.6.3 Conclusion of the immobilization

The recombinant His-tagged CpSADH was successfully immobilized with two different methods: entrapment and covalent binding via adsorption. In the first method, the enzyme was entrapped in PVA beads. Without further characterization, the functionality of the immobilized enzyme in PVA beads was determined. The immobilized enzyme in PVA remained protected against the organic solvent and was active within the hydrogels. Acetophenone was converted by the immobilisate and about 132 μmol phenylethanol was produced. In the second method, the enzyme was covalently bound on two different types of Sepabeads: EC-EP and EC-HFA. The former has epoxy group as reactive group and the latter has epoxy-amino group as the reactive group. The immobilized enzyme showed activity both in pure aqueous system and also in aqueous-organic two-phase system. The use of two-phase system is particularly important for substrates (here acetophenone), which otherwise exhibit only very small water solubility. Here the organic phase serves as reservoir for the hydrophobic substrate and leads for an extraction of the product and therefore lead to higher yields. The small aqueous phase contained the cofactor, which unlike the PVA beads was not possible to co-immobilize together with the enzyme. Under these conditions, the enzyme showed very good conversion with the EC-HFA Sepabeads and about 230 μmol phenylethanol was built. The conversion with the EC-EP Sepabeads was very poor and as low as only 14 μmol phenylethanol was produced. The stability of the immobilized enzyme on EC-HFA Sepabeads was 284 h which was approximately same like that of the soluble enzyme (in one phase) at a temperature of 25 °C. But with an increase in temperature (30 and 37 °C), the stability of the immobilized enzyme got higher than that of the soluble enzyme (approximately 1.5 times). Furthermore, when compared to the half-life of the native enzyme in aqueous-organic two-phase system with the same phase ratio of 1:3 (buffer: hexane), it was found that the immobilized enzyme on the EC-HFA Sepabeads was much more stable under the same phase ratios and the half-life increased to approximately 3.6 times.

4 Summary

The (*S*)-specific NADH-dependent carbonyl reductase from *C.parapsilosis* DSMZ 70125 strain (CPCR) is a very versatile biocatalyst and because of its broad substrate spectrum, the possibility of cofactor-regeneration and high stereo-selectivity, very much interesting for the chemoenzymatic synthesis of pharmaceuticals and agrochemicals. However, the wtCPCR was present in the yeast cells only in very small amounts (< 0.03% of the total cell proteins). In the year 2001, Bönitz identified the partial amino acids sequences of the wtCPCR and thereby cloned and expressed this protein in *E.coli*. But the recombinant enzyme did not show similar substrate spectrum like the wtCPCR (results obtained from JFC). Another (*S*)-specific NADH-dependent secondary alcohol dehydrogenase that was first isolated from *C.parapsilosis* IFO 1396 (CpSADH) by a Japanese research group showed also broad substrate specificities. However, the recombinant CpSADH was not characterized and was basically employed for whole-cell biocatalysis for the oxidation of 1,3-butanediol.

This research work was aimed at cloning and expression of the carbonyl reductase from *C.parapsilosis* (CPCR) followed by characterization and immobilization of the recombinant enzyme. With this view, the first step was to identify which of the two alcohol dehydrogenases that were described to be present in two different strains of *C.parapsilosis* was the real CPCR. For this, using the information of the partial amino acids sequences available for the wtCPCR, the gene coding for the CPCR was amplified by PCR based genome walking from the gDNA library of *C.parapsilosis* DSMZ 70125 strain. The derived amino acids sequence showed that it contained a total of 403 amino acids, which were 54 additional amino acids on the N-terminal as compared to the amino acids sequences of the CPCR that had been described before. Analysis of this additional amino acids sequence on the N-terminal showed that it was not a signal-peptide sequence but rather a part of the mature protein. Interestingly it was found that the recombinant CPCR was not active in *E.coli* expression strains just after 2 expression cycles. Changes of expression parameters gave no positive results. Inclusion bodies were not found either. The enzyme showed no activity. Due to this problem in the expression of the recombinant CPCR (cloned in this work) in *E.coli*, a recombinant CPCR preparation (X-Zyme) which is also based on the available partial amino acids sequences of the wtCPCR was used for necessary comparison. The CpSADH gene was amplified also from the same gDNA library of *C.parapsilosis* DSMZ 70125 strain via conventional PCR with primers deduced from the published sequences. The amplification of

the CpSADH and the CPCR genes from the same strain of *C.parapsilosis* confirmed that at least these two ADHs were present in the same strain (“CPCR” type strain of *C.parapsilosis*). Further identification was done by modeling the active sites of the CpSADH and the CPCR (based on their amino acids sequences) which showed that the CpSADH would accept more bulky substrates than in comparison to the CPCR. Experimental analysis of some potential substrates based on the prediction of the model, showed that methyl-3-oxovalerate (described to be converted by CPCR) was converted by the recombinant CpSADH (both with and without His₆-tag) and also by the wtCPCR (JFC) but not by the recombinant CPCR (X-Zyme). Comparison of the substrate spectrum of the recombinant CpSADH showed that the enzyme had very similar conversion rates like the commercially available wtCPCR (JFC), but a very different conversion rate with the substrates were observed with the recombinant CPCR (X-Zyme). The modeling information, substrate spectrum, special characteristics of the recombinant CpSADH identified that it was indeed the CpSADH which was the “Real CPCR”.

The His-tagged CpSADH showed higher specific activities than the one without any His₆-tag, and possessed a specific activity of 653.7 U/mg, 82.3% yield (after IMAC purification and ultrafiltration). Enzymatic syntheses with the recombinant His-tagged CpSADH were carried out in one-phase system, aqueous-organic two-phase system, gel-stabilized two-phase system and also with immobilized enzyme in Sepabeads in two-phase system. In all the systems, acetophenone was used as the model substrate and the cofactor NADH+H⁺ was regenerated with 2-propanol. The highest product concentration was obtained in a liquid two-phase system with 25% (v/v) hexane as substrate reservoir, and 103 mM of (*S*)-1-phenylethanol was produced (51.5% conversion). In gel-stabilized two-phase system with PVA-immobilized biocatalyst, 44.1 mM (*S*)-1-phenylethanol was formed. Among the epoxy-carrier Sepabeads (EC-EP and EC-HFA), the immobilized enzyme in a two-phase system showed a very high activity on the EC-HFA (57.6 mM phenylethanol produced) but a very low activity on the EC-EP (3.5 mM phenylethanol produced) due to the hydrophilic and hydrophobic natures of the respective Sepabeads. The stability of the enzyme immobilized on EC-HFA (in two-phase system) was higher than the soluble enzyme (in pure aqueous medium) at higher temperatures (30 and 37 °C) and also higher than that of the soluble enzyme with the same aqueous-organic phase ratios at 25 °C. Therefore concerning the productivity, the EC-HFA Sepabeads was indeed better than that of the PVA matrix though the stability of the latter was not studied.

5 References

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