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Biocatalytic Carbon Nitrogen Double Bond Reduction

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

vorgelegt von

Diplom Lebensmitteltechnologe

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CHAPTER 1: INTRODUCTION

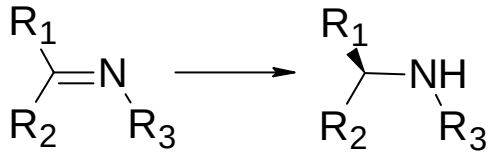
1.1 Chirality and biocatalysis

The biological activity of a given chiral compound results usually from the stereochemistry of the molecule. Thus, while one enantiomer shows a desired therapeutic effect, the other isomer can have no, or even an opposite effect. In this area, probably the most well known example is the commercial Contergan®, containing the active substance thalidomide. Whereas the (*R*)-enantiomer provides a beneficial effect, the (S)-enantiomer 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 1992. Safety information is now demanded for individual stereoisomers of products submitted for approval for commercialization. Although racemates will still be continued to be approved on a case-by-case basis, detailed information on both enantiomers is required (Peters, 1998).

Several strategies have been developed for the production of those valuable chiral compounds. Although those compounds can be produced by chemical synthesis, usually the aid of a catalyst is crucial for the achievement of high enantiopurities. In this area, biocatalysis – using either whole cells or isolated enzymes – represents a powerful toolbox of approaches for the efficient production of those chiral compounds. This 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. 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, 1999). Yet, that expected increased industrial implementation of biocatalytic uses may be hampered, or retarded, by many other factors, not directly related to scientific aspects. A recently published review provides a more realistic viewpoint on the actual situation in industrial biocatalysis (Hilterhaus, 2007).

From a practical viewpoint, biocatalysts offer some advantages over chemical catalysts. These include the possibility of performing processes under rather mild reaction conditions, which usually leads to the avoidance of unwanted by-products (especially when isolated enzymes are used) (Liese, 1999). Moreover, as an asset for biocatalysis, aspects like high chemoselectivity, regioselectivity and especially stereoselectivity for the production of enantiomerically pure compounds must be pinpointed. Those attractive features are not necessarily exhibited by the chemical catalysts, though impressive development has been reported in this field during the last decades. Within biocatalysis, one of the core approaches is that of resolution of racemates. Such a strategy makes use of the selectivity of the enzymes for one of the enantiomers of a given chiral molecule, whereby the other enantiomer remains virtually unrecognized. Notably, modern biocatalytic approaches in which the non reacted enantiomer is *in situ* racemized have recently appeared (the so-called dynamic kinetic resolutions). These developments enhance even more the attractiveness that the herein reported biocatalytic tools can have for practical performances, as theoretical yields of 100% can be achieved. Notably, the fact that nowadays enzymes can be cloned and overexpressed, allows the production of tailor-made enzymes, especficially envisaged for a certain chemical application. Taken together, those developments confer biocatalysis a promising horizon of uses and applications, expected to occur in the forthcoming years.

As a result of the growth in demand for chiral compounds, the market for asymmetric building blocks is growing fast. This trend has provided an enormous impetus for the development of enantioselective chemical and biochemical transformations. In this regard, although biocatalytic applications of all possible enzyme classes have been reported in literature (Liese, 2006) there is still room for developments, since many enzymatic platforms need still to be developed to a practical concept. Focusing on this need, in particular the present study aims to explore the possibility of producing chiral secondary amines via asymmetric reduction of prochiral imines. (Scheme 1.1). This enzymatic approach has not yet been developed at wide extent, and thus only few academic reported cases can be found in the open literature (Li, 2004; Vaijayanthi, 2008).

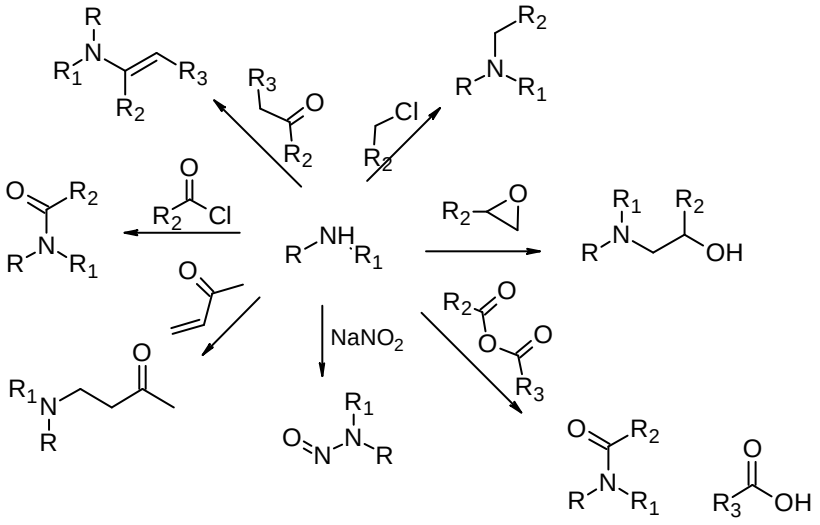


Scheme 1.1: Synthesis of chiral secondary amines via asymmetric reduction of prochiral imines.

1.2: Chiral secondary amines

1.2.1: Industrial production of chiral secondary amines

Secondary chiral amines are interesting products for the chemical and pharmaceutical industry. They can be a final product, but also versatile commodities and building blocks for their further chemical derivatization. An overview of those chemical routes to generate added value products from amines is depicted in scheme 1.2.

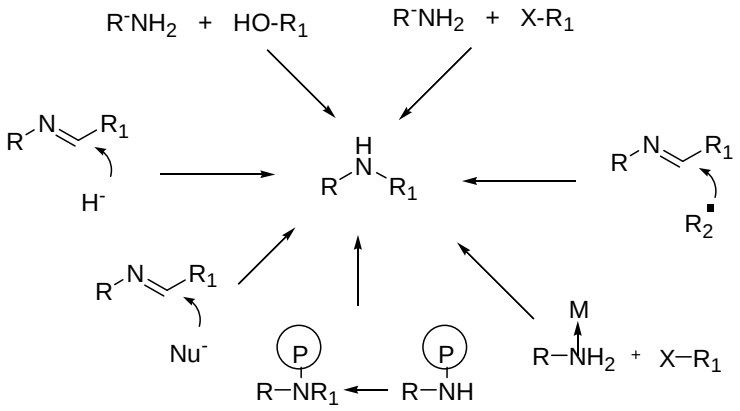


Scheme 1.2: Possible pathways for the organic further derivatization of secondary amines

Presently, the production of optically active secondary amines at industrial scale relies only in chemical methods. Thus, no alternative biocatalytic routes have been established so far for this type of products. The chemical methods for the production of chiral secondary amines are mainly direct hydrogenation of imines precursors, or hydrogenation of cyanogroups, leading to the corresponding amines. This latter strategy is particularly

useful for both the production of primary or secondary amines (Breuer, 2004; Salvatore, 2001).

Despite the fact that organic synthesis can offer several routes for accessing chiral secondary amines on a lab-scale, few processes are reported on an industrial scale. The most of these synthetic strategies are illustrated in scheme 1.3.

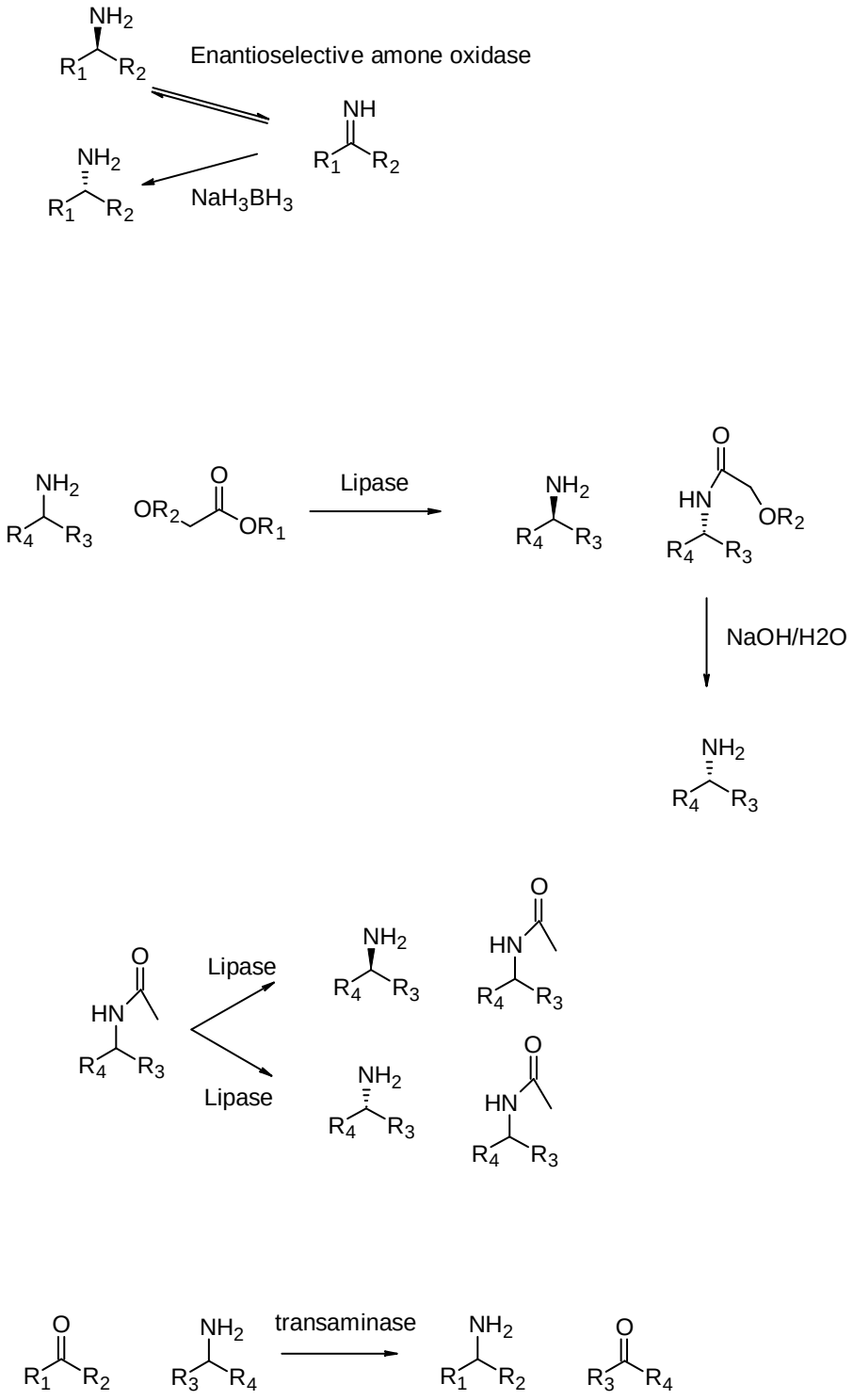


Scheme 1.3: Strategies for the chemical synthesis of secondary amines

Taking into account the relevance that chiral amines have in synthetic purposes, and the apparent lack of biocatalytic routes to afford such compounds in a practical and enantiopure manner, the present work has focused on the prospect to find an alternative route for the production of such secondary amines, in an attempt to broaden the platforms for the production of amines nowadays existing.

1.2.2: Enzymatic production of chiral amines

The current state-of-the-art of biocatalytic production of chiral amines comprises only a handful of processes, illustrated in scheme 1.4. Most of them are for the production of primary amines. Only one of those strategies produces chiral secondary amines, by means of a chemo-enzymatic step.



Scheme 1.4: Biocatalytic reactions for the production of optically active amines

For the formation of primary amines one of the most studied biocatalytic routes is the direct amination of carbonyl groups using transaminases (Cho, 2003). Yet, this route has two major drawbacks: firstly, aspects on thermodynamics are unfavourable to the amine

formation. Thus, to enhance the yield of the overall process, the product should be removed *in situ*, to drive the reaction toward the synthesis. Secondly, an amine donor is required, thus making cumbersome the reaction's control and the downstream process to purify the desired product from the reaction's mixture (Kim, 2007).

A different process that successfully runs on tons scale is followed by BASF for the production of some amines. Among the produced amines some are used as crop-protectants, others as chiral resolving agents for chemical synthesis. In their strategy, the racemic primary amine is acetylated via conventional chemical synthesis method. Later on, an enantioselective lipase is used to solve the racemic mixture (Riechers, 2000; Ditrich, 2000).

An alternative to these processes relies on the possibility to follow a chemo-enzymatic approach. The racemic amine is oxidized via a monoaminooxidase and the imine produced enzymatically is later reduced *in-situ* by a enantioselective chemical catalyst (Alexeeva, 2002).

The same research group managed to produce also chiral secondary amines following the same strategy (Carr, 2005) by engineering the biocatalyst (the monoaminooxidase).

As it can be noticed, at the moment no processes for the production of chiral secondary amines via direct reduction of the imine precursors have been estabilished. This route may be very attractive, as some of the disadvantages reported for the other biocatalytic routes might be overcome, especially the low thermodynamic yield in the case of amines produced by mean of transamination, or the multistep acylation-deacylation and isolation of the product in the case of the BASF route with lipases. Therefore, in the present study the attempts made so far to exploit this possible strategy will be illustrated in detail.

1.3: Anaerobic bacteria and anaerobic respiration

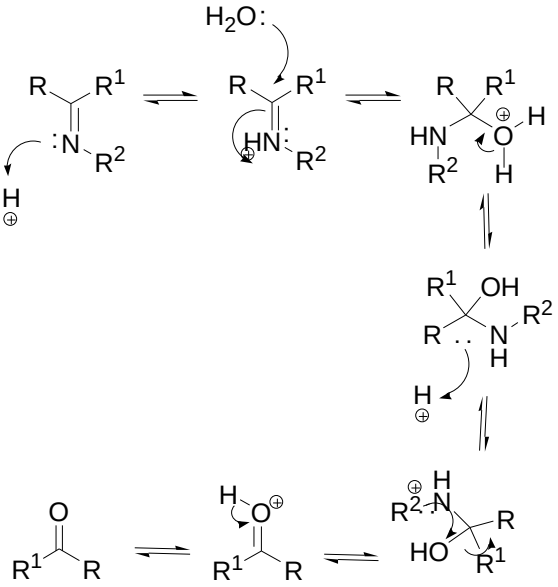
Anaerobic bacteria possess different metabolism compared to the aerobic ones. The ultimate difference is that anaerobic bacteria cannot use molecular oxygen as electron acceptor of the electrons produced during the "anaerobic respiration" (Madigan, 2005). Prompted by this observation, a research group published a study (Li, 2004) describing a specific anaerobic microorganism (*Acetobacterium woodii*) able to reduce the C=N bond of an imine as a possible way to dispose of the electrons coming from the anaerobic metabolism.

The stated observation that imines where reduced only when the organism was grown with caffeic acid as inducer, led to the conclusion that the enzyme responsible for the reduction of caffeic acid was able to perform also the reduction of the imine. This aspect fits in a current important concept in biocatalysis, that of enzymatic promiscuity.

1.4: Promiscuity of enzymes

The most of the molecules bearing imine do not come from natural sources, but are rather the (by-)products of man-made chemical synthesis. The most of the imines are not present in nature because the C=N bond is not stable in water, but suffers nucleophilic addition of water on the double bond, thus leading to spontaneous self-hydrolysis of the molecule (Clayden, 2001). The process is shown in scheme 1.5.

Based on this simple observation, the conclusion that nature in its evolutionary history could not have evolved an enzyme for this purpose was drawn by us in the beginning of the project.



Scheme 1.5: The mechanism of spontaneous imine hydrolysis in water.

Nevertheless, biocatalysis has the potential to perform even reactions that are not existing in nature, relying on the so called “promiscuity concept” (Kazlauskas, 2005). In this respect, a promiscuous catalytic activity is the ability of a single active site to catalyse

more than one chemical transformation. These transformations may differ in the functional group involved, that is, the type of bond formed or cleaved during the reaction, and/or may differ in the catalytic mechanism or path of bond making and breaking. It is also interesting to notice that the promiscuous activity of an already existing enzyme is the base for the evolution of a molecular level (Tawfik, 2006), allowing an organism to be fit for "new" environmental conditions with its "old" enzymatic machinery.

The challenge of imine reduction via biocatalysis to the correspondent chiral secondary amines could be view as a problem of identifying the right class of enzymes that can lead to a biocatalytic imine reduction as promiscuous activity. Prompted by this concept, the quest about which microorganisms and / or enzymes could perform such a reduction of iminic bonds focused on two possible candidates: enoate reductases and carbonyl reductases

1.5: Enoate reductases

Enoate reductases (E.C. 1.3.1.31) are enzymes that catalyze the reduction of C=C bonds. These enzymes can perform the reduction using either NADH+H+ or NADPH+H+ as cofactors. However, usually NADH+H+ is preferred (Simon, 1991). The reaction's mechanism involves the transfer of a hydride ion (H-) on the partially positive carbon atom of the carbon carbon double bond (Snape, 1997).

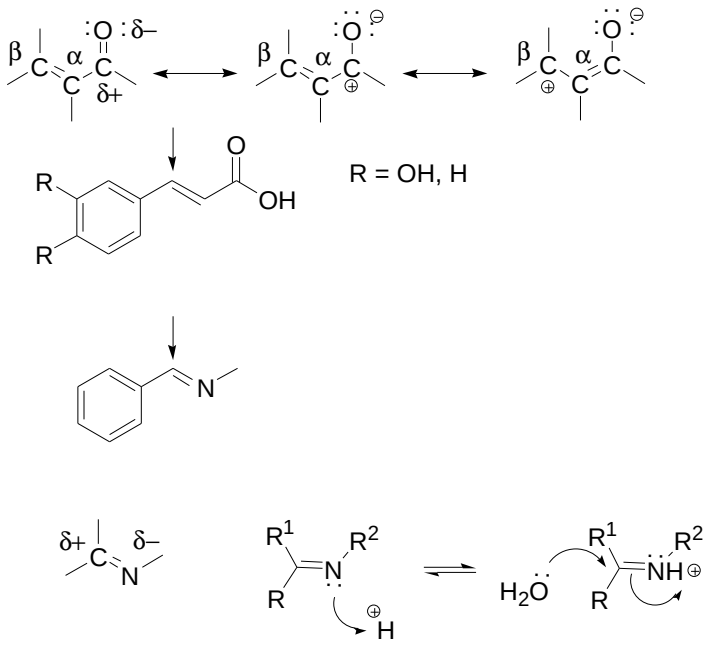
Up to now only few enzymes belonging to this family have been characterized or cloned. Reasons for that lack of results can be found in the fact that they are not widespread in nature. In addition, they contain an iron-sulfur cluster – crucial for the enzymatic performance –, that is unstable in the presence of molecular oxygen. Notably, enoates are widely accepted by different anaerobic bacteria as terminal electron acceptor in the so called "anaerobic respiration" (Madigan, 2005)

Both imines and enoates bear unsaturated bonds, so the postulation that the enoate reductases could reduce imines looked reasonable, and was reputed worth of further investigation.

In particular, caffeic acid is reduced by *Acetobacterium woodii* in this respiration process, and a research group published a paper (Li, 2004) where they stated that this microorganism is able to utilize imines as electron acceptor in this kind of respiration, thus leading to the reduction of the iminic bond.

One of the imines mentioned in the paper has striking similarities with two enoates that are widely reduced by enoate reductases, as shown in scheme 1.6.

For these reasons enoate reductases were tested as possible imine reductases, either as whole wild type cells biocatalysts, or as cloned and expressed in *Escherichia coli*.



Scheme 1.6: Similarities with enoates and imines. In detail are shown the ketoenolic tautomerization and the electronegativity of the nitrogen atom in the iminic bond. The carbon atom highlighted by the arrow in the enoate is partially positive and constitutes the site of attack by the hydride ion (H^-) during the reduction reaction's. The carbon atom highlighted by the arrow in the imine is the one where the postulated attack by the hydride ion (H^-) of the enzyme could take place.

In the figure 1.1 the similarities in the electron distribution between the caffeic acid and the benzylidenmethylamine are showed. In both the molecules the regions shadowed in light blue represents the regions with lack of electrons, thus being possibly the regions where the H- transferred by the enoate reductase could attack, and in both the molecules these electron deficient (regions correspond to the carbon atom highlighted by the arrow in the scheme 1.6).

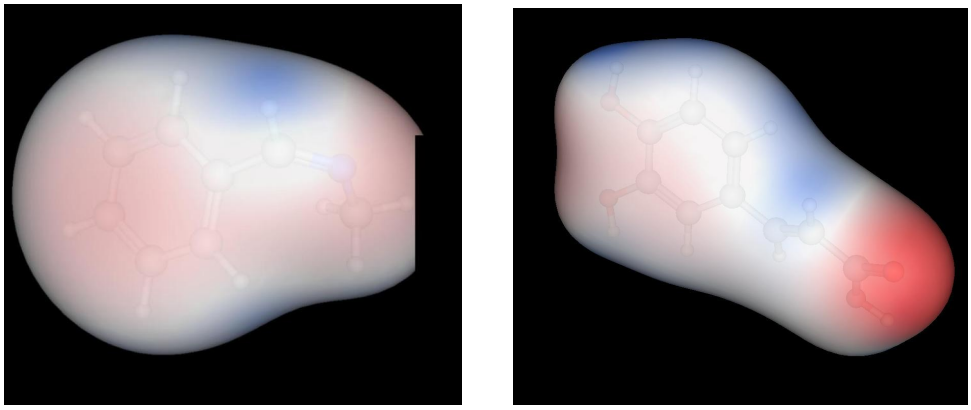


Figure 1.1: Similarity in the electron's distribution in the postulated promiscuous substrate for enoate reductase (benzylidenmethylamine, on the right side) and the natural substrate for the enoate reductase (caffeic acid, left side). The regions shadowed in light blue are the ones with the highest electron deficiency; the ones shadowed in red highlight the highest electron density.

1.6: Carbonyl reductases

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 $NADH+H^+$ and/or $NADPH+H^+$ as cofactors. They are also a class of enzymes exploited by industries as robust tool to obtain chiral alcohols (De Wildeman, 2007).

Eevn in this case, as for the enoate reductases, the reaction's mechanism involves the transferement of a hydride ion (H^-), but in this case the hydride ion is transferred directly to the oxygen of the carbonyl group.

At first sight carbonyl groups do not appear closely related to imines. Nevertheless, there is an interesting analogy to chemical catalysis. Intriguingly, chemical catalysts that reduce carbonyl groups can sometimes, under specific reaction conditions, reduce iminic bonds (Tang, 2003). When instead chemical catalysts that reduce C=C bonds, do not reduce C=N bonds under any reaction condition.

Prompted by this analogy with chemical catalysis, recombinant carbonyl reductase from Candida boidinii [CPCR; E.C.1.1.1.1] was chosen in this study as target for the reduction attempts of imines. It has been chosen because it 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 other alcohol

dehydroganases, most substrates are reduced at higher rates by the CPCR, especially the reduction of synthetic useful acetophenone derivatives and 4-chloro-3-oxobutanoate (Peters, 1993). The CPCR showed the possibility of converting acetophenone and many of its derivatives to the corresponding (S)-phenylethanol in NADH-dependent catalysis, which is interesting because it shows a opposite enantioselectivity in respect of the already available ADHs. The CPCR was also chosen for several practical reasons. Among them, because was recently cloned in our research group (Dr. Bhattacharjee, PhD thesis, 2006) and shown to be a robust catalyst for the reduction of carbonyl groups under different reaction conditions.

1.7: Metagenomic DNA

The analysis of the "metagenome" is fueling the biocatalysis, in terms of isolation of new enzymes (Streit, 2004). For this reason, the metagenome has been considered in this project a source to mine in the attempts to isolate new enzymes that could be able to reduce imines. As quite a couple of carbonyl reductases have already been described and are available for this research, the focus of this approach was put on enoate reductases.

The metagenome is the total genomic material recovered from a specific environment. The metagenome is considered a promising genetic source for retrieving active biocatalysts, as well as sequence and environmental information (Schmeisser, 2007).

The metagenome can be mined in different ways to recover new enzymes. Mainly two different approaches can be followed: "sequence based screening" and "activity based screening" (Gabor, 2007), as showed in scheme 1.7.

In the first approach sequence information of the desired enzyme are needed; the primary sequence of selected enzymes are aligned with the help of bioinformatics databases, and regions containing high homology of amino acid residues are identified. Degenerate primers to amplify via PCR those regions can be designed and the metagenomic DNA is used as template to run PCRs.

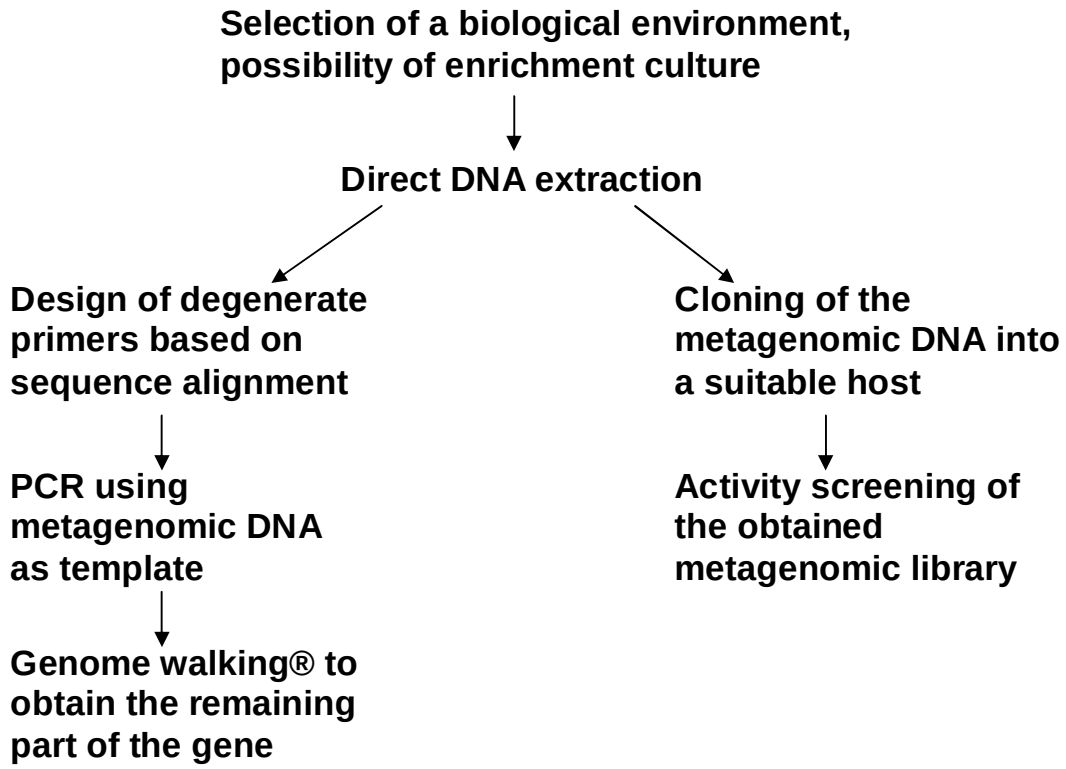
The approach via activity based screening requires the screen of a "metagenomic library". This is a genetic library obtained by inserting genes recovered from a metagenomic DNA extraction into a suitable host. The resulting library can be screened by different techniques, for example in the "high throughput assays" (Reymond, 2006) or with the help of visual screening (hydrolysis of turbid substrates leading to clarification's aloes) or using selective media. In the case of high throughput screening or visual screening, the

sensitivity of the screening technique has fundamental importance: as the cloning vectors for metagenome do not overexpress the foreign proteins, the codon usage and the promoters are not optimized, the total level of the desired protein actively folded can be very low. In this case using a non-optimized or intrinsically non-sensitive enough screening technique, many biocatalysts present in the cloned DNA strands can be missed.

Screening metagenomic libraries for new enzymes presents several advantages *vs*. traditional methods of isolation of new biocatalysts, but also bottlenecks at the same time. Among the advantages the most impressive is to partially solve the problems of the so called “plate count anomaly” (Streit, 2004). Currently today, only ca. 1-5 % of the total biodiversity found in nature can be cultivated in laboratory under standard microbiological methods. This means that the traditional cell culture screening based on commercial microbiological sources (e.g. DSMZ in Germany, ATCC in U.S.A.) or on isolation of new organisms from the environment, neglects the most of the biodiversity, thus leading to the discovery of enzymes that have been maybe already characterized.

Screening a metagenomic library enhances the probability of retrieving a new non-characterized biocatalyst. Moreover the isolation of a completely new and non-characterized enzyme can lead in many cases to strong IP positions, which obviously makes this approach more attractive from economic viewpoints.

The drawback of the technique is that the host, in which the metagenomic genes are cloned, can be not optimal to express the foreign protein, in terms of folding, promoter effect, protein level and toxicity of foreign protein.



Scheme 1.7: Flow-sheet about the alternative approaches for the metagenomic screening. On the left side the "sequence based screening" that doesn't require the construction of a metagenomic library, but simply uses the metagenomic DNA as template for PCRs.
On the right side the so called "activity screening", that requires the construction of a metagenomic bank to screen the metagenomic genes into a suitable host.

1.8: Aim of the present studies

The overall research aim of this project is to explore the possibility of reducing iminic bonds by means of biocatalysis Since no biocatalytic imine reduction platform is nowadays present, it has been postulated that this fact represented a chance to expand the actual biocatalytic toolbox. To achieve this goal, it has been thought to address to the enzymatic promiscuity concept, thus it could be attained identifying the class of enzymes that could reduce the carbon nitrogen double bond as "promiscuous activity" (Kazlauskas, 2005).

The identification of two enzyme classes that could perform this bioreduction has been made (enoate reductases and carbonyl reductases) based on structural studies, mechanism studies and analogies with already reduced substrates.

The enoate reductase from C*lostridium acetobutylicum* was cloned and overexpressed, based on an already published study (Rohdich, 2001), moreover the isolation of a

complete new enoate reductase from a metagenomic library obtained cloning the DNA of the anaerobic digestor of a paper industry has been achieved. These two enzyme were applied in the promiscuous reduction of imines.

The carbonyl reductase from *Candida parapsilopsis* was applied also in the biocatalytic imine reduction, due to its broad substrate range and high turnover number, but also because via molecular modeling it has been showed that the imine substrate could dock and coordinate inside the active site of this specific reductase.

CHAPTER 2: MATERIALS AND METHODS

2.1: Materials and Devices

All strains have been purchased by DSMZ (Germany) when not present in the Microbial Collections of the RWTH Aachen Institute.

The used devices are indicated in the text.

All the reagents and chemicals have been supplied by Sigma-Aldrich (Germany) and used without further purification, if not otherwise specified.

N-Benzyl-methyl amine was synthesized, since it was not commercially available.

2.1.1: Synthesis of N-Benzylmethyl acetamide

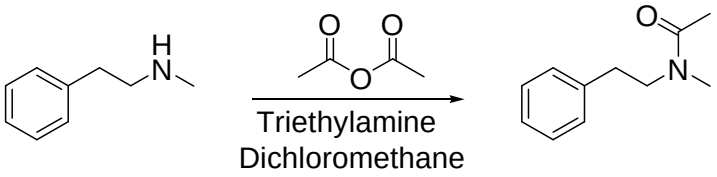


Figure 2.1: Synthesis of N-Benzylmethyl acetamide

To a solution of 3.0 mL N-Benzylmethyl amine (20.9 mmol) in 100 mL dichloromethane, 2.8 mL triethylamine (20.9 mmol) was added and the mixture was cooled to 0˚C with an ice bath. 2.0 mL of acetic anhydride was added dropwise and the reaction stirred overnight at room temperature. Then, the organic phase was washed with HCl aq. solution (pH= 4.5, 3 x 100 mL), $NaHCO_3$ sat. aq. solution (3 x 100 mL) and brine (1 x 100 mL). The organic phases were collected and dried using Na_2SO_4 and concentrated *in vacuo*. 2.4 g (14.0 mmol) of light yellow solid was recovered, corresponding to an overall yield of 67%.

^{1}H-NMR ($CDCl_3$, 400 MHz, Varian): δ 7.24 (t, 2H, Ph), 7.14 (d, 2H, Ph), 7.07 (d, 1H, Ph), 3.61 (t, 2H, -CH_2-N), 2.92 (s, 3H, -N-CH_3), 2.79 (t, 2H, -CH_2-CH_2-), 2.01 (s, 3H, -CO-CH_3).

2.2: Cultivation media and protocols

All media for *Escherichia coli* Top10, BL21(DE3) and DH5α cultivation, namely LB-medium, TB medium and M9 medium were prepared following standard protocols

(Sambrook, 1989). The media for the cultivation of any other strains (yeasts, *Lactobacilli*, *Clostridia*) as well as *Acetobacterium woodii* and *Sporomusa termitida* were based on protocols described by DSMZ, Germany.
Solidified agar plates were made with 1.5% agar weight per volume (w/v).

2.2.1: Cultivation media and protocols for *Escherichia coli* strains

LB medium

Tryptone 10 g/L
NaCl 5 g/L
Yeast extract 5 g/L
Deionized water as required
The pH was adjusted to 7.2 with NaOH 1M. The solution was autoclaved for 21 minutes at 121°C.

TB medium

Solution I
Glycerol 4 mL
Tryptone 12 g
Yeast extract 24 g
Deionized water to 900 mL
Solution II
KH_2PO_4 2.31 g
K_2HPO_4 12.54 g
Deionized water to 100 mL
The two solutions were autoclaved for 21 minutes at 121°C separately, then cooled at circa 50 °C and mixed to obtain the final medium.

M9 (mineral media)

Mineral solution M9 10x
Na_2HPO_4 60.00 g in 1000 mL deionized water
KH_2PO_4 30.00 g in 1000 mL deionized water
NH_4Cl 10.00 g in 1000 mL deionized water
NaCl 5.00 g in 1000 mL deionized water
Glucose 20% solution

Glucose 20.00 g in 100 mL deionized water.

$MgSO_4$ 1M solution

$MgSO_4$ 12.00 g in 100 mL deionized water.

$CaCl_2$ 0.1M solution

$CaCl_2$ 1.10 g in 100 mL deionized water.

Proline 20 mg/mL

Proline 0.2 g in 10 mL deionized water.

Thiamine 1M

Thiamine-HCl x 2 H_2O 3.37 g in 10 mL deionized water.

The final media was obtained by mixing the required amounts of different solutions sterilized for 21 minutes at 121°C separately in autoclave and bringing to final volume of 1 L with deionized steril water.

Mineral solution M9 salts 10 x	100 mL
$MgSO_4$ 1M	1.0 mL
$CaCl_2$ 0.1M	1.0 mL
Glucose	10 mL
Proline	0.02 g
Thiamine-HCl 1 M	1.0 mL
Deionized water	

Thiamine and proline stock solutions were obtained by filter sterilizing the solutions through sterile filters.

2.2.2: Cultivation medium and protocol for *Acetobacterium woodii*

Acetobacterium woodii DSMZ 1030 medium

Composition:

NH_4Cl	1.00 g
KH_2PO_4	0.33 g
K_2HPO_4	0.45 g
$MgSO_4$ x 7 H_2O	0.10 g
Trace element solution	20 mL
Vitamin solution	20 mL
Yeast extract	2.00 g
Fructose	10.00 g

Resazurin	1.0 10^{-3} g
$NaHCO_3$	10.00 g
Cysteine-HCl x H_2O	0.50 g
Na_2S x 9 H_2O	0.50 g
Deionized water	1.0 L

Preparation procedure:

The ingredients, except fructose, cysteine and sodium sulfide, were dissolved and the medium autoclaved at 121°C for 21 minutes. After cooling, the medium was sparged with nitrogen for 30 minutes and brought inside the anaerobic bench. Fructose, cysteine and sodium sulfide were filter sterilized and added as anaerobic sterile stock solutions. Before using the media, the pH was adjusted to 8.2 by adding sterile anaerobic NaOH 1M.

Sterile anaerobe caffeic acid solution was added to the media (10 mL of 0.1M stock solution pro liter media) when the induction of caffeic acid reductase was needed.

Trace element solution

Composition:

Nitrilotriacetic acid	1.50 g
$MgSO_4$ x 7 H_2O	3.00 g
$MnSO_4$ x 2 H_2O	0.50 g
NaCl	1.00 g
$FeSO_4$ x 7 H_2O	0.10 g
$CoSO_4$ x 7 H_2O	0.18 g
$CaCl_2$ x 2 H_2O	0.10 g
$ZnSO_4$ x 7 H_2O	0.18 g
$CuSO_4$ x 5 H_2O	0.01 g
$KAl(SO_4)_2$ x 12 H_2O	0.02 g
H_3BO_3	0.01 g
Na_2MoO_4 x 2 H_2O	0.01 g
$NiCl_2$ x 6 H_2O	25 10^{-3} g
Na_2SeO_3 x 5 H_2O	0.3 10^{-3} g
Deionized water	1 L

Preparation procedure:

Nitrilotriacetic acid was dissolved in water, the pH was adjusted to 6.5 with KOH 1M, then the minerals were added. Then the pH was adjusted to 7.0 by adding KOH 1M.

Vitamin solution

Composition:

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine-HCl	10.0 mg
Thiamine-HCl x 2 H_2O	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
D-Ca-pantothenate	5.0 mg
Vitamin B_{12}	0.1 mg
p-Aminobenzoic acid	5.0 mg
Lipoic acid	5.0 mg
Deionized water	1 L

Preparation procedure:

The ingredients were dissolved in deionized water, the solution was sparged for 30 minutes with nitrogen and later filter sterilized in the anaerobic bench.

Caffeic acid 0.1M stock solution

A fresh solution of caffeic acid was prepared before the use. 0.18 g of caffeic acid was suspended in 5 mL deionized water and 1mL NaOH 1M was added, the solution vortexed for 5 minutes and, after complete dissolution of caffeic acid, the pH was adjusted to 7.0 by addition of NaOH or H_3PO_4 1M. The neutrality of the solution was checked with indication paper and then the solution was brought to a final volume of 10 mL with deionized water. The solution was sparged with nitrogen for 5 minutes and filter sterilized in the anaerobic bench.

2.2.3: Cultivation medium and protocol for *Sporomusa termitida* DSMZ 4440

Composition:

K_2HPO_4	0.35 g
KH_2PO_4	0.23 g
NH_4Cl	0.50 g
$MgSO_4$ x 7 H_2O	0.50 g
$CaCl_2$ x 2 H_2O	0.25 g

NaCl	2.25 g
$FeSO_4$ x 7 H_2O	2 10^{-3} g
Vitamin solution	10.0 mL
Dithiothreitol (DTT)	1 10^{-3}M
Trace element solution SL-10	1.0 mL
$NaHSeO_3$	10^{-7} M
Yeast extract	2.00 g
Casitone	2.00 g
$NaHCO_3$	4.00 g
Resazurin	1.0 10^{-3} g
Deionized water	1 L

Preparation procedure:

The components were dissolved in deionized water, the pH was adjusted to 7.0 and autoclaved at 121°C for 21 minutes. After cooling, the medium was flushed with nitrogen for 30 minutes and was brought inside the anaerobic bench. DTT and vitamin solution were added to the medium from an anaerobic filter sterilized stock solution.

The vitamin solution was the same used for *Acetobacterium woodii*.

Trace element solution SL-10

Composition:

HCl (25%; 7.7M)	10.00 mL
$FeCl_2$ x 4 H_2O	1.50 g
$ZnCl_2$	0.07 g
$MnCl_2$ x 4 H_2O	0.1 g
H_3BO_3	6 10^{-3} g
$CoCl_2$ x 6 H_2O	0.19 g
$CuCl_2$ x 2 H_2O	2.00 10^{-3} g
$NiCl_2$ x 6 H_2O	0.02 g
Na_2MoO_4 x 2 H_2O	0.04 g
Deionized water	1L

Preparation procedure:

$FeCl_2$ was dissolved first in HCl, later diluted with deionised water and the other salts were dissolved into it. The solution was sparged for 30 minutes with nitrogen and later filter sterilized in the anaerobic bench.

2.2.4: Cultivation medium and protocol for *Clostridium celerecrescens*

Components:

$(NH_4)_2SO_4$	1.30 g
KH_2PO_4	1.50 g
K_2HPO_4 x 3 H_2O	2.90 g
$MgCl_2$ x 6 H_2O	0.20 g
$CaCl_2$ x 2 H_2O	0.07 g
$FeSO_4$ x 7 H_2O	1.2 10^{-3} g
Trace element solution SL-10	1.000 mL
Resazurin	1.0 10^{-3} g
Yeast extract	2.00 g
Cellobiose	6.00 g
Deionized water	1 L

Preparation procedure:

The medium was prepared by mixing all the components in the deionized water, then the pH was adjusted to 6.0 with HCl 1M and autoclaved at 121°C for 21 minutes. After cooling, the medium was sparged with nitrogen for 30 minutes and brought inside the anaerobic bench, where the pH was adjusted to 7.2 with sterile anaerobic NaOH 1M. Cellobiose was autoclaved separately at 121°C for 21 minutes and added to the medium after having adjusted the pH to 7.2.

2.2.5: Cultivation medium and protocol for yeasts

The yeast's collection of RWTH Institute was cultivated using the universal medium for yeasts.

Composition:

Yeast extract	3.00 g
Malt extract	3.00 g
Peptone	5.00 g
Glucose	10.00 g

Deionized water to 1 L

Preparation procedure:

All the elements were dissolved in deionized water and the solution was autoclaved at 121°C for 21 minutes.

Biomass preparation of the yeasts:

Pre-culture tubes were prepared inoculating 5 mL liquid yeast medium with the correspondent cryostocks and grown at the specific optimal temperature (30°C or 37°C for 24-48 hours). The biomass for the screening for imine reduction or benzaldoxime reduction was prepared inoculating 1 mL of liquid broth from the pre-cultures into 50 mL of liquid broth in 250 mL Erlenmeyer flasks. In the case the biomass was used to test benzaldoxime reduction, also 0.5 mL of filter sterilized of 0.1M stock solution of benzaldoxime as inducer was added in the liquid media before the inoculum.

2.2.6: Cultivation medium and protocol for *Lactobacillus* species

Composition:

Peptone	10.00 g
Beef extract	10.00 g
Yeast extract	5.00 g
Dextrose	20.00 g
Ammonium citrate	2.00 g
Sodium acetate	5.00 g
$MgSO_4$	0.10 g
$MnSO_4$	0.05 g
K_2HPO_4	2.00 g

Deionized water to 1 L

Preparation procedure:

All the ingredients were dissolved with deionized water, then the pH was adjusted to 5.0 with HCl 1M, then distributed in Erlenmeyer flasks and autoclaved at 121°C for 21 minutes.

Pre-culture tubes were prepared inoculating 5 mL liquid yeast medium with the correspondent criostocks and grown at the correspondent temperature (30°C or 37°C for 24-48 hours). The biomass for the screening for imine reduction or benzaldoxime reduction was prepared inoculating 1 mL of liquid broth from the pre-cultures into 50 mL of liquid broth in 250 mL Erlenmeyer flasks. In the case the biomass was used to test benzaldoxime reduction, also 0.5 mL of filter sterilized of 0.1M stock solution of benzaldoxime as inducer was added in the liquid media before the inoculum.

2.2.7: Cultivation medium and protocol for *Clostridia*

Composition:

Yeast extract	5.00 g
Glucose	50.00 g
Asparigine	2.00 g
$(NH_4)_2SO_4$	2.00 g
NaCl	1.00 g
$MgSO_4$	0.40 g
$MnSO_4$	0.01 g
$FeSO_4$	0.01 g
KH_2PO_4	0.75 g
K_2HPO_4	0.75 g

Deionized water to 1 L

Preparation procedure:

All the ingredients were dissolved in deionized water, then the pH was adjusted to 6.0 with HCl 1M, then distributed in Schott flasks and autoclaved at 121°C for 21 minutes. After cooling, the medium was sparged with nitrogen for 30 minutes and brought inside the anaerobic bench.

All handlings and inoculations of *Clostridia* have been performed inside the anaerobic bench. Pre-culture tubes were prepared inoculating 5 mL liquid medium with the correspondent cryostocks and grown at the correspondent temperature (30°C or 37°C for 24-48 hours). The biomass for the screening for imine reduction or benzaldoxime reduction was prepared inoculating 1 mL of liquid broth from the pre-culture into 50 mL of liquid broth in 50 mL sterile falcon tubes and incubated in anaerobic jars for 24-48 hours at the desired temperature. In case that biomass was used to test benzaldoxime reduction, also 0.5 mL of filter sterilized of 0.1M stock solution of benzaldoxime as inducer was added in the liquid media before the inoculum.

2.2.8: Cultivation medium for enrichment of the environmental sample

Composition:

Bactopepton	1.00 g/L
NH_4Cl	2.00 g/L
NH4OH	1.00 g/L

KH_2PO_4	0.75 g/L
K_2HPO_4	0.75 g/L
Yeast extract	1.00 g/L
Vitamin solution	20.00 mL/L
Mineral solution	20.00 mL/L
Sodium acetate	2.00 g/L
Sodium pyruvate	2.00 g/L
Sodium formiate,	2.00 g/L

The medium was prepared by mixing all components except the vitamin solution (see section 2.2.2), the pH was adjusted to 7.2 with NaOH 1M and autoclaved at 121°C for 21 minutes. After cooling, the medium was sparged with nitrogen for 30 minutes and brought inside the anaerobic bench. Vitamine solution was added as anaerobic sterile stock solution.

In the anaerobic bench 5 g of the environmental sample (the anaerobic sludge of a paper industry) were transferred in sterile 50 mL falcon tubes, they were then filled with the media for the enrichment, sealed and transferred on an orbital shaker at 30°C for 48 hours.

2.3: Molecular biology methods

2.3.1: Preparation *of Acetobacterium woodii* genomic DNA (gDNA)

The total genomic DNA from of *Acetobacterium woodii* DSMZ 1030 was isolated using the DNeasy tissue kit from QIAGEN (Hilden, Germany) following manufacturer suggestions. The quality of the obtained DNA was checked running 5 µL of the obtained gDNA on a 0.8% agarose gel stained with ethidium bromide (0.5 µg/mL).

2.3.2: Preparation of *Sporomusa termitida* and *Clostridium celerecrescens* genomic DNA (gDNA)

The total genomic DNA from *Sporomusa termitida* DSMZ 4440 and from *Clostridium celerecrescens* DSMZ 5628 were isolated by harvesting cells at the late logarithmic growth phase. Cells were lysed as described in the literature (Di Salle, 2006) by employing a buffer containing two detergents, namely Triton X-100 and N-lauryl sarcosine at a final concentration of 0.12% and 1.6% respectively. Cells were centrifuged for 2 minutes in a microcentrifuge at 14,500 rpm. The cell pellet was resuspended in TEN-buffer (Tris/HCl

10mM, EDTA 1mM, NaCl 150mM, pH 8.0). An equivalent amount of TENST (TEN-buffer supplemented 0.12% Triton X-100 and 1.6% N-lauryl sarcosine) was added and the mixture was incubated for 30 minutes at room temperature.

The resulting supernatant contained proteinaceous components and cellular DNA. The mixture was extracted with an equivalent volume of phenol/chloroform/isoamylalcohol (24:24:1). The biphasic system was mixed by vortexing and the phases were separated by centrifugation for 10 minutes in a micro-centrifuge at maximum speed. The aqueous phase was extracted two more times and then DNA was precipitated by the addition of 2.5 volumes of cold 96% ethanol. The precipitated DNA was collected at the bottom of Eppendorf tubes by centrifugation at 14,500 rpm for 20 minutes at room temperature. The resulting supernatant was discarded, the pellet washed with cold 70% ethanol and centrifuged again as described above. After discarding the supernatant, the DNA pellet was dried in a laminar flow in the clean bench for 1 hour.

The obtained gDNA pellet was dissolved in TE buffer, containing 2.5 mg/mL DNase free RNase (Boehringer Mannheim, Germany). Generally 5 µL of this genomic DNA was used for restriction endonucleolytic analysis.

2.3.3: Metagenomic DNA extraction from enriched cultures

Falcon tubes containing 5 g of the environmental sample from an anaerobic digestor of the paper industry were incubated for 48 hours on a shaker at 120 rpm at 30°C during an enrichment of the biomass and the desired activity as described in section 2.2.8; then the falcon tubes were centrifuged for 30 minutes at 4,600 rpm at 4°C.

The solid pellet was transferred aerobically to a mortar, frozen with liquid nitrogen and grinded manually for 5 minutes. The obtained paste was extracted following the Zhou method (Zhou, 1996). To this end, an amount of 13.5 mL of DNA extraction buffer (DEP, see below) and 100 µL of proteinase K (10 mg/mL) was added to the cell paste. The obtained dense solution was transferred in sterile SS34 tubes and incubated for 30 minutes at 37°C (horizontal shaking at 225 rpm). After the incubation, 1.5 mL of 20% SDS was added and the samples were incubated in a water bath at 65°C for 2 hours with gentle end-over-end mixing every 15 to 20 minutes. The supernatants were collected after centrifugation at 6,000 x *g* for 10 minutes at room temperature for separating the water phase from the solid debris of the matrix and transferred into 50 mL sterile falcon tubes.

The remaining pellet was extracted two more times by adding 4.5 mL of the extraction buffer (DEP) and 0.5 mL of 20% SDS. The mixture was vortexed for 10 seconds,

incubated at 65°C for 10 minutes and centrifuged as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1, vol/vol).

The aqueous phase was separated from the phenol/chloroform/isoamyl alcohol by centrifugation at 4°C for 5 minutes at 4,600 rpm carefully without disturbing the layer of denatured proteins at the interface. The DNA was precipitated from the aqueous phase by addition of 0.6 volumes of cold isopropanol followed by incubation for 1 hour at 4°C. A pellet of crude nucleic acids was obtained by centrifugation at 4,600 rpm for 1 hour at 4°C, washed with cold 70% ethanol, centrifuged again as described above. The resulting supernatant was discarded and the DNA pellet dried in the sterile laminar flow in the clean bench for 1 hour. The dried DNA pellet was resuspended in sterile MilliQ water and 5 µL were loaded on a 0,8 % agarose gel to evaluate the yield and quality of the DNA preparation.

In order to understand if the additional grinding step used in the above described method increased the overall gDNA yield, a parallel metagenomic DNA extraction was performed using an aliquot of the same enriched sample and employing the previously described Zhou method (Zhou, 1996), but without grinding the sample in a mortar with liquid nitrogen. The results are discussed in the section 3.3

DEP

Composition:

Tris	0.1M
Na_2-EDTA	0.1M
Na_2HPO_4	0.1M
NaCl	1,5 M
CTAB	1 % (w/v)
pH 8.0	

2.4: Construction of libraries

2.4.1: Cloning of *Acetobacterium woodii* DSMZ 1030 genomic DNA into *E.coli*

The genomic DNA of *Acetobacterium woodii* DSMZ 1030 was cloned into the PWE15 vector as described by Henning (Henning, 2006).

Briefly, the gDNA was partially digested with restriction enzyme *Sau3A*I (cohesive-end cutter). The reaction's mixture was run on a 0.8% agarose gel, fragments of about 5-10 kb were purified with QIAGEN gel-extraction kit (QIAGEN, Hilden, Germany) and ligated into dephosphorylated PWE15 cloning vector which was previously digested with *BamH*I.
The following paragraphs describe in details the construction of the Acetobacterium woodii library in *E.coli.*

2.4.2: Construction of *Acetobacterium woodii* genomic DNAlibrary into *E.coli*

The DNA of *Acetobacterium woodii* was isolated following the method described in the section X . The estimated size of the isolated DNA was ca. 20 kb. The DNA revealed to be smeared, probably due to shear forces in the pipetting steps. Anyway, the purity was assumed to be high since in all the further cloning steps neither inhibition of restriction, nor of ligation enzymes was observed.
The genomic DNA of *Acetobacterium woodii* was subjected to restriction with enzyme Sau3AI. The digested DNA was run through a preparative agarose gel and the bands corresponding to the desired molecular weight (ca. 7-10 kb) were excised and recovered with the QIAGEN gel recovery kit. The obtained DNA was ligated to PWE15 vector, subsequently digested with restriction enzyme BAMHI, and dephosphorylated with CIAP (calf intestine alkaline phosphatase), to minimize the number of religants.
Later on, the ligation mixture (20 µL) was desalted by adding double distilled sterile water to final volume of 200 µL, and filtered using microcon centrifuge tubes. The dilution and filtration were repeated two times. The desalted reaction mixture was recovered from the microcon centrifuge tube, diluted again to 20 µL and transformed in *E.coli* JM 109 (DE3).

2.4.3: Transformation of *Acetobacterium woodii* library

The ligation mixture was transformed in *E.coli* JM 109 (DE3) electrocompetent cells. Typically, 5 µL of the reaction mixture were added in a 1,5 mL eppendorf tubes containing 100 µL of cells. The tubes were incubated on ice for 30 minutes, transferred in pre-chilled 2 mm gap electroporation cuvette and pulsed with BIORAD gene pulser apparatus. After the pulse (200 Ω, 2,5 kV, 3-5 milliseconds) 1 mL of SOC medium was added and the mixture transferred to a 1,5 mL sterile eppendorf tube. The tube was shaken at 37°C in a thermomixer for circa 40-45 minutes, to allow the regeneration of the plasmids. After this incubation time, the content of the eppendorf was centrifuged and resuspended in 300 µL

of LB medium, and 150 µL of this mixture were plated on a solid LB plate containing 100 µg/mL of ampicilline.
The yield in clones was ca. 200-300 clones per plate, thus 400-600 clones with 5 µL of ligation mixture.
The quality of the library, in terms of average insert size and the frequency of clones harboring *Acetobacterium woodii* DNA within each library, was determined by restriction analysis of 24 plasmids isolated from randomly chosen clones as described in section 2.4.6. The restriction analysis indicated that 70% of the selected clones carried an insert and the insert size was ca. 9 kb. This means that ca. 57 Mb of *Acetobacterium woodii* DNA were inserted in *E.coli* and subjected to further screening for the caffeic acid reduction activity in High Throughput format.

2.4.4: Cloning of metagenomic DNA into *E.coli*

The metagenomic DNA was blunted using T4 DNA polymerase (MBI Fermentas, Germany) at room temperature following manufacturer instructions.
The resulting blunt ended DNA product was purified using the PCR purification kit (QIAGEN, Hilden, Germany) and resuspended in double distilled sterile water.
The obtained DNA was digested with the restriction enzyme *EcoR*V and run on a 0.8% agarose gel. Fragments of about 4-7 kb in size were purified using the QIAGEN gel-extraction kit and ligated into the pZero® vector (Invitrogen, USA) which was previously digested using *EcoR*V as described in the manual (p-Zero Background® cloning kit, Invitrogen USA).
In a typical ligation reaction a molar ratio vector:insert of about 1:10, and an end volume of 20 µL was used.

2.4.5: Construction of metagenomic DNA library into *E.coli*

In order to enhance the overall DNA yield, a modification of the Zhou protocol was performed. The modification consisted in freezing the environmental sample with liquid nitrogen and later grinding it in a sterilized mortar, as described in the section 2.3.3 of materials and methods.
The improvement in the overall yield due to the grinding with liquid nitrogen was estimated in being at least 10 times higher.
The obtained DNA was blunted with Taq polymerase in a thermocycler (Eppendorf, Germany), as described in a standard protocol (MBI Fermentas, Germany). The obtained

blunted DNA was subjected to restriction with the blunt-cutter ECORV and run through a preparative agarose gel. The bands corresponding to a molecular weight in the range 4-7 kb were excised and the DNA was extracted out of the gel with the kit “DNA gel extraction kit” (QIAGEN) according to manufacturer suggestions.
The obtained metagenomic DNA fragments were ligated into the *Eco*RV site of the high-copy plasmid vector pZero-2 (Invitrogen, USA), using a 1:10 vector:insert ratio.
The ligation mixtures (30 µL) was desalted diluting it to 300 µL with double distilled sterile water and filtered using microcon centrifuge tubes. The operation was repeated two times. The desalted reaction mixture was recovered from the microcon centrifuge tube, diluted again to 20 µL and transformed in *E.coli* JM 109 (DE3).

2.4.6: Transformation of the metagenomic library

The ligation mixture was transformed in *E.coli* JM 109 (DE3) electrocompetent cells. Typically, 5 µL of the reaction mixture were added in a 1,5 mL eppendorf tubes containing 100 µL of cells. The tubes were incubated on ice for 30 minutes, transferred in pre-chilled 2 mm gap electroporation cuvette and electroporated with a BIORAD gene pulser apparatus (BIORAD, Germany). After the pulse (200 Ω, 2,5 kV, 3-5 milliseconds) 1 mL of SOC medium was added and the mixture transferred to a 1,5 mL sterile eppendorf tube. The tube was shaken at 37°C in a thermomixer (Eppendorf, Germany) for circa 40-45 minutes, to allow the regeneration. After this incubation time, the content of the eppendorf was centrifuged and resuspended in 300 µl of LB and 150 µL were plated on a solid LB plate with 50 µg/mL kanamycin.
The yield in clones was circa 150-200 clones per plate, thus 300-400 clones with 5 µL of ligation mixture.
The quality of the obtained library was evaluated as described in the section 2.4.6.

2.4.7: Enoate reductase recovery from the metagnomic DNA and other DNA sources via PCR amplification

The metagenomic DNA obtained as described in section 2.3.3, as well as gDNA of *Acetobacterium woodii* and *Sporomusa termitida*, was used as template in PCR amplifications in the attempts to isolate a new enoate hydrolase, following a published protocol (Uchiyama, 2006).
One primer set (“Degenerate PCR”) was designed for the primary gene amplification, the second set (“Nested PCR”) was designed for the nested PCR. The primers used are given

in table 2.1. The primers were designed based on the alignment of already published enoate reductases, as cleared in section 3.4.

Table 2.1: Primers used for the primary metagenomic DNA isolation

	Degenerate PCR	Nested PCR
Forward primer	CERF1 5- GAR MGI GCI AAR GGI GGI ACI GG -3	CERF2 5- GCI GGI TTY GAY GGI GTI SAR ATH CAY GC -3
Reverse primer	CERR1 5- TCR CAI CCI ACI ARI CCI CCI CC-3	CERR2 5- GCY TCV MWI CCI GCI ACI CCI CCI CC -3

PCR amplifications were carried out using Ampli-Taq™ DNA-Polymerase (Applied Biosystems, USA) under standard amplification conditions as exemplified in manual of the supplier. The thermal cycling protocol was as follows: 10 minutes of initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 minute, 45 seconds annealing at 50°C and 1.5 minutes of elongation at 72°C, concluded by an final elongation for 10 minutes at 72°C (table 2.2). Successful PCR-amplification was verified by agarose-gel electrophoresis and PCR products of the correct size were purified using a commercial gel-extraction kit (Eppendorf, Germany).

The rest of the gene was obtained from a Genome Walker™ (Clontech, USA) following the strategy published by Uchiyama (Uchiyama, 2006).

Table 2.2: PCR conditions for the amplification of conserved regions of enoate reductases.

Steps	**Time**	**Temperature**	**Cycles**
Denaturation	10 minutes	95 °C	1
Denaturation	1 minute	95°C	40
Annealing	45 seconds	50°C	
Elongation	1.5 minutes	72 °C	

Final elongation	10 minutes	72°C	1
Cooling	∞	4°C	--

2.4.8: Transformation of plasmids in *Escherichia coli* cells via electroporation

In general, ligation mixtures were transformed in *E. coli* by electroporation, using 2 mm electroporation cuvette (BIORAD, Germany) and a Gene-Pulser apparatus (BIORAD, Germany). The ligation mixtures containing the *Acetobacterium woodii* gDNA in PWE15 were transformed in *E. coli* JM109 (DE3) as host, while the ligation mixtures containing metagenomic DNA in pZero® were transformed in *E.coli* TOP 10 cells. Typically, 5-10 µL of the respective ligation mixtures, or correspondingly 10-20 ng of the circular vector DNA, were added to 100 µL of thawed electrocompetent *E.coli* cells and mixed by gentle tapping of the Eppendorf tube. The mixture was incubated on ice for about 30 minutes. After the incubation the cells were transferred with a pipette to a prechilled electroporation cuvette. DNA take-up by the cells was induced by applying an electric pulse (200 Ω, 2,5 kVolts, 3-5 milliseconds) by using the Gene- Pulser apparatus (BIORAD, Germany) (in *E.coli* standard settings). After the pulse, 1 mL of sterile SOC medium was added to the cuvette. The broth was transferred to a sterile 2 mL Eppendorf tube and incubated in a thermomixer at 37°C for 45 minutes at 1200 rpm for the regeneration. After the regeneration the mixture was plated on solid LB broth containing the appropriate antibiotic (ampicillin for the *Acetobacterium woodii* library, kanamycin for metagenomic DNA library).

2.4.9: Transformation of chemically competent cells by heat shock

About 10 µL of the ligation mix, or correspondingly 10-20 ng of the circular vector DNA, was added to 190 µL of thawed competent cells and mixed by gentle tapping of the microcentrifuge tubes. This mix was incubated on ice for 30 minutes and then a heat shock was applied by incubating the cells at 42°C for 90 seconds. Cells were placed back on ice and incubated for additional 5 minutes. To the transformation mixture 1 mL of sterile SOC medium was added. The cells were then incubated at 37°C for 45 minutes at 1200 rpm in a thermo-mixer (Eppendorf, Germany). The transformation mixture was plated onto LB agar plates containing the appropriate antibiotic.

2.4.10: Plasmid isolation

Plasmids of interest were isolated using the Eppendorf Mini plasmid-isolation kit. Typically, 1.5 mL of overnight LB-grown *E.coli* strains (for high copy number vectors), or 3.0 mL (for medium copy number vector such as PWE15) were processed following manufacturer's suggestions.

2.4.11: Quality evaluation of the prepared libraries

24 single clones for each library were picked from the transformation plates and cultivated overnight in LB-broth supplemented with the appropriate antibiotic at 37°C. The plasmids were isolated as previously described. The plasmids were double digested with two restrictions enzymes flanking the multiple cloning site of the vector. Typically, 3 µL of plasmid-DNA were transferred in a sterile 1.5 mL Eppendorf tube, 0.5 µL of each restriction enzyme, 1 µL of digestion buffer and 5 µL of double distilled sterile water were mixed and incubated at 37°C for 1 hour. After the digestion, 2 µL of loading buffer were added and the whole mixture run on a 0.8% agarose gel stained with ethidium bromide (0,5 µg/ml). For determination of fragment size a defined amount of DNA size marker (GeneRuler™ 1 kb DNA Ladder) was loaded on the gel. After the gel was run at 100 Volts for 45 minutes, bands were visualized on a UV transilluminator (Eagle-Eye II, Stratagene, USA).

2.4.12: Random transposon insertion

The plasmid pEPH (recovered from the clone able to grow on the selective media for epoxide hydrolase) was subjected to a random transoposon insertion using the Epicentre transoposon insertion kit (Epicentre, USA) by following manufacturer's instructions.

2.4.13: DNA restriction digestion

Digestion of the DNA with restriction endonucleases was performed in the buffer supplied with the restriction enzyme in accordance with the suppliers' recommendations. All the restriction endonucleases were bought from MBI Fermentas (Germany). Mostly digestion was done for 4-5 hours (in case of *EcoR*I, *Pst*I and *Hind*III) or up to 16 hours (in case of *Nde*I and *Xho*I) using 10-20 units (*EcoR*I, *Pst*I and *Hind*III) or 5-6 units (*Nde*I and *Xho*I) of the enzyme and 0.5-1.5 µg DNA, respectively. The digestion reaction was incubated for the appropriate time at 37°C and analyzed by agarose gel electrophoresis. For preparative restriction digestions e.g., for cloning of DNA fragments, the resulting reaction mixture was

purified with the PCR purification kit (Qiagen, Hilden, Germany) and quantified by agarose gel electrophoresis.

2.4.14: 5' Dephosphorylation of DNA fragments

The digested and linearized cloning vector PWE15 was dephosphorylated at its 5'-termini directly after restriction digestion using 1 unit of Calf Intestine Alkaline Phosphatase (CIAP, MBI Fermentas) for 60-90 minutes at 37°C in accordance with the suppliers recommendations. The resulting linearized and dephosphorylated plasmid DNA was purified with the PCR purification kit (Qiagen, Hilden, Germany).

2.4.15: PCR amplifications of the gene of the Putative epoxide hydrolases

In order to clone and express the putative epoxide hydrolase identified in the metagenomic screening, different sets of PCR oligonucleotides were designed (Table 2.3). One primer set, set a in table 2.1 (Hydro_*Nde*I_fw and Hydro_Stop_*Xho*I_rev), was used to amplify the putative EH including a stop-codon at the 3'-end of the gene, which resulted after cloning into pET22b in the expression of the native enzyme without addition of a purification-tag. To allow easier purification of the expressed protein a second primer set, set b in table 2.1 (Hydro_*Nde*I_fw and Hydro_*Xho*I_rev), was used, which does not contain a stop-codon and thus allowed, after cloning in pET22b, the addition of a C-terminal Hexa-Histidine-Tag.

Table 2.3: Primer sets for the amplification of the Putative epoxide hydrolase gene

	Set a	Set b
Forward primer	Hydro_*Nde*I_fw 5- TCC AGT ACA TAT GCG CTA TCC CTT TCT CGG TTA TGA AGC C-3	Hydro_*Nde*I_fw C5- TCC AGT ACA TAT GCG CTA TCC CTT TCT CGG TTA TGA AGC -3
Reverse primer	Hydro_Stop_*Xho*I_rev 5-TAA CAT CTC GAG TCA GAG CCG CCG GGA TGA AAA GG	Hydro_*Xho*I_rev 5- TAA CAT CTC GAG GAG CCG CCG GGA TGA AAA GG-3

PCR amplifications were carried out using Taq™ DNA-Polymerase (MBI Fermentas, Germany) under standard amplification conditions as exemplified in manual of the supplier. The thermal cycling protocol was as follows: 2 minutes of initial denaturation at

95°C, followed by 30 cycles of denaturation at 95°C for 1 minute, 45 seconds annealing at 57°C and 2 minutes of elongation at 72°C, concluded by final elongation for 5 minutes at 72°C (table 2.4). Successful PCR-amplification was verified by agarose-gel electrophoresis and PCR products of the correct size were purified using a commercial gel-extraction kit (Eppendorf, Germany).

Table 2.4: PCR conditions for the amplification of the gene of the Putative epoxide hydrolases

Steps	Time	Temperature	Cycles
Denaturation	2 minutes	95 °C	1
Denaturation	1 minute	95°C	30
Annealing	45 seconds	57°C	
Elongation	2 minutes	72 °C	
Final elongation	5 minutes	72°C	1
Cooling	∞	4°C	--

2.4.16: Cloning of PCR product of the gene of the putative epoxide hydrolase

The respective PCR products, amplified as described above, were digested with *Nde*I and *Xho*I as described in the manual of the manufacturer. Digested PCR products were cloned into similarly hydrolyzed pET22b+ and transformed into *E.coli* JM109 (DE3) for expression. Correct cloning was verified by restriction analysis and sequencing of the insert in pET22b+. DNA sequencing was carried out at MWG biotech (Germany) from both directions using vector specific primers.

2.5: Reaction setup for the low throughput screening

2.5.1: Reaction setup for the low throughput screening of imines with microbial collections

The cells were resuspended, anaerobically when necessary, in phosphate buffer pH 7.0, 0.1M ($NaHPO_4/K_2HPO_4$) and transferred to a glass vial; the solution was overlayed with an equivalent amount of n-hexane in which the imine chosen as substrate was dissolved to 10mM final concentration. After incubation (24-48 hours) at 30°C an aliquote of the n-hexane was injected in the GC and the presence of product (secondary amine) was detected using the retention time of commercially available standards as references. The

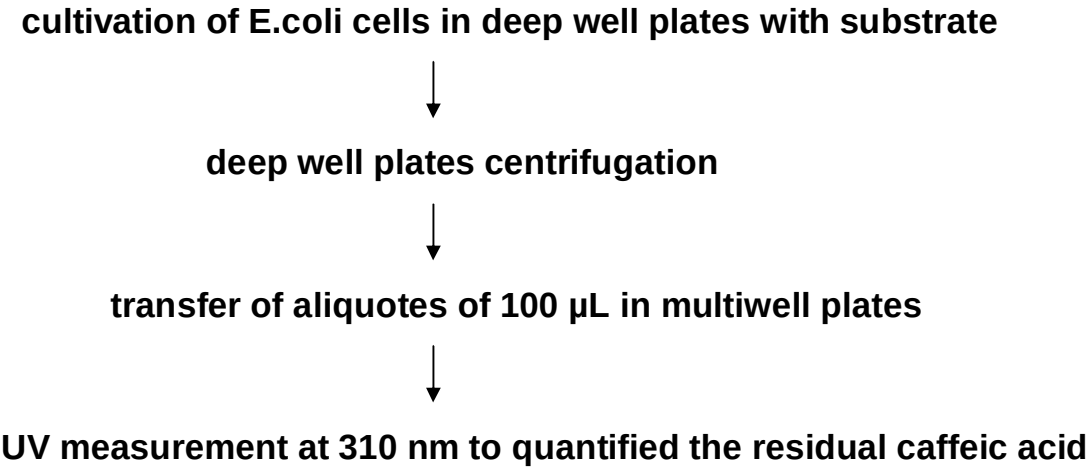
pH of the remaining water phase was raised to about 12 with NaOH 2M, extracted twice with an equal amount of ethyl acetate, dried under nitrogen flow, resuspended in n-hexane and injected into the GC.

2.5.2: Reaction setup for the low throughput screening of benzaldoxime with microbial collections

The cells were resuspended, anaerobically when necessary, in phosphate buffer pH 7.0, 0.1M ($NaHPO_4/K_2HPO_4$) containing benzaldoxime 10mM and transferred to eppendorf tubes. A solution of different carbon sources (fructose, glucose, glycerol, sucrose, maltose, ribose, xylose and piruvic acid at the concentration of 2.0 g/L) was added to the reaction mixture to provide the expected reaction with the necessary potential cofactor. The reactions were monitored after 24, 48 and 72 hours incubation in an orbital shaker at 37°C. The eppendorf tubes were centrifuged in a microfuge, 14.500 rpm for 5 minutes and the cleared supernatant was filtered and analyzed via HPLC.

2.6: High throughput screening for caffeic acid reductases

E.coli cells propagating the *Acetobacterium woodii* and metagenomic DNA libraries were grown in deep well microtiter plates. Each well was inoculated with a single clone using the Genetix colony picker robot (Genetix, England). The medium was the terrific broth, supplemented with caffeic acid 0.8 10^{-3}M and the antibiotic specific for selection (ampicillin 0.1 mg/mL for the library of *Acetobacterium woodii*, kanamicin 0.05 mg/mL for the metagenomic library); after the inoculation the plates were sealed with rubber lids. Air consumption generated by cell respiration allowed the establishing of anaerobic conditions in the microtiter plates. After 48 hours of growth at 30°C in a shaker, the microtiter plates were centrifuged at 4,000 rpm for 20 minutes and transferred to the TECAN pipetting workstation (United Kingdom). A 0.1 mL aliquote of clear supernatant was transferred in disposable transparent microtiter plates and read at 310 nm in a multiwell spectrophotometric reader. The whole procedure is summarized in the scheme 2.1.



Scheme 2.1: Summary of the developed High Throughput Screening for caffeic acid reduction.

Since a positive control test was not available, a calibration curve was assayed using different dilutions of caffeic acid in the used media. It has been demonstrated that this substrate has a very high molar extinction coefficient, allowing the screening of the whole library with very low concentrations of caffeic acid (0,8 10^{-3}M); due to the high reproducibility of the robot applied in the aliquoting steps, the detection of conversion as low as 10% (ca. 0.08 10^{-3}M) was possible.

2.7: Screening for epoxide hydrolases

2.7.1: Colony assay for epoxide hydrolases

The colonies, which remained in the transformation plates after the robotic cell handling (section 2.6), were washed away with sterile phosphate buffer, pH 7.0, 0.1M.

1.5 mL of each suspension containing the isolated clones was centrifuged at 14,500 rpm for 2 minutes and washed twice more with the same buffer. 10 µL from each transformation plate was used to inoculate assay tubes for the epoxide hydrolases screening.

2.7.2: Selective media for epoxide hydrolase screening

Glycidol (2,3-epoxy-1-propanol) was added at 0.05% (w/v) final concentration to Luria Bertani broth (LB) supplied with the specific selecting agent (kanamycine) Only clones

able to hydrolyze the toxic compound were able to grow, thus giving turbidity in the essay tubes after 24-48 hours incubation in a orbital shaker at 30°C.

2.7.3: Screening of the random transposon insertion minilibrary for epoxide hydrolase positive clone

Single clones from the transformation plates were manually picked and inoculated in selective media for epoxide hydrolase screening and in LB media as control.

2.8: Carbon nitrogen double bond bioreduction by CPCR

2.8.1: Imine reduction by Candida parapsilopsis carbonyl reductase (CPCR) in buffer

Benzylidenmethylamine was dissolved in triethanolamine (TEA) buffer, pH 7.0, 0.1M, at 10mM final concentration. Reactions were performed in 1 mL mixtures in UV-transparent disposable cuvettes adding 10 μL of recombinant purified CPCR, 10 μL of 200 mM stock solution of NADH in TEA buffer, 800 μL of imine substrate in TEA buffer preheated at 30°C. The reactions were monitored for 5 minutes in a UV-Vis spectrophotometer at 340 nm at 30°C. After monitoring the initial velocity, NADH was further added (40 μL of 200mM stock solution) and the reactions prolonged overnight in a thermomixer at 30°C.

2.8.2: Imine reduction by Candida parapsilopsis carbonyl reductase (CPCR) in hexane

E.coli cells expressing the recombinant and active CPCR were thawed and distributed in glass vials (1 g of wet cells per vial). The pellets were resuspended in 4 mL of different buffers (in the pH range from 4-13). Lysozyme was added to a final concentration of 1 mg/mL and the cell suspension was incubated on ice for 30-40 minutes. The mixtures were sonicated using four cycles of 1 minute bursts with 70% intensity and with 1 minutes cooling period between each burst. The preparation was frozen at -80°C in the glass vials and later lyophilized. The reaction started in the vials by adding 4 mL of hexane containing the substrate to be tested (benzylidenmethylamine for the reaction and acetophenone as the positive control, both at 10mM final concentration) and isopropanol at the same concentration (10mM) for cofactor recycling.

2.8.3: Imine reduction by Candida parapsilopsis carbonyl reductase (CPCR) in biphasic system water/organic solvent

30 μL of purified CPCR of a stock solution (20 units/ml) were added to 920 μL of reaction's buffer in a GC glass vial. The reaction was started by adding 50 μL of a 200mM NADH stock solution and overlaying the water phase with 1 mL hexane containing 10mM of benzylidenmethylamine. After incubation at fixed temperature and time (typically 25-30-37-45°C for 6-18-24-48 hours) the hexane phase was transferred to a new GC vial and analyzed via GC. The pH of the water phase was increased to 12 with NaOH 1M and extracted twice with an equal amount of ethyl acetate. The organic phase was dried under nitrogen flow, the obtained dried matter resuspended in 200 μL hexane and analyzed via GC.

2.8.4: Benzaldoxime reduction by Candida parapsilopsis carbonyl reductase (CPCR) in buffer

Benzaldoxime was dissolved in triethanolamine (TEA) buffer, pH 7.0, 0.1M, at 10mM final concentration. Reactions were performed in 1 mL mixtures in UV-transparent disposable cuvettes adding 10 μL of recombinant purified CPCR, 10 μL of 200mM stock solution of NADH in TEA buffer, 800 μL of imine substrate in TEA buffer preheated at 30°C. The reactions were monitored for 5 minutes in a UV-Vis spectrophotometer at 340 nm at 30°C. After monitoring the initial velocity, NADH was further added (40 μL of 200mM stock solution) and the reactions prolonged overnight in a thermomixer at 30°C. Aliquots of the reaction setup were withdrawn and incubated on a thermomixer at 25°C after addition of NADH up to 2mM. At different times, the reaction mixtures were centrifuged and filtered, then analyzed via HPLC.

2.9: Carbon nitrogen double bond bioreduction by enoate reductases

2.9.1: Production of recombinant enoate reductases

Plasmid of pET22b+ containing the gene of Clostridium acetobutylicum enoate reductase (CaERI) or the metagenomic enoate reductase (MERI) were transformed in *E.coli* BL21 (DE3) or *E.coli* JM109 (DE3) containing already a plasmid for the expression of glucose dehydrogenase resistant on kanamycin. Precultures of 5 mL in LB media (ampicillin 0.1 mg/mL, kanamycin 0.05 μg/mL) were grown overnight at 37°C. 2 mL of cell suspension were used to inoculate 100 mL of TB (0.1 mg/mL ampicillin, 0.05 mg/mL) in 250 mL schott flasks closed with aereation lids. The cells were grown aerobically at 37°C to an optical

density of 1 (typically in 5-6 hours), later cooled to 25°C, induced with the investigated amount of Isopropyl-β-D-thiogalactopyranoside (IPTG) and sparged with nitrogen for 15 minutes. After the flasks were made anaerobe, they were incubated at 25°C overnight (16 hours). The cells were pelleted by centrifuging them at 4,600 rpm for 20 minutes, washed with sterile anaerobe phosphate or citrate-phosphate buffer (at the required pH, 0.1M).

2.9.2: Imine reduction by recombinant enoate reductases in water solution

The obtained biomasses of *E.coli* BL21 (DE3) or *E.coli* JM109 (DE3) overproducing the recombinant glucose dehydrogenase and enoate reductase of *Clostridium acetobutylicum* (CaERI) as well as the enoate reductase from the metagenome (MERI) were diluted with anaerobe potassium/sodium 0.1M phosphate buffer pH 7.0 to an optical density of about 3.0. The reactions were started by adding 500 μL of cell suspension to a freshly prepared anaerobe solution of benzylidenphenylamine 20mM and glucose 40mM for cofactor recycling in 2 mL eppendorf tubes. The tubes were incubated at fixed temperature and time (typically 25-37-45°C for 6-18-24-48 hours). After each incubation time, the pH of the water phase was immediately raised to about 12 with NaOH 1M and extracted twice with an equal amount of ethyl acetate. The reaction products in the organic phase were dried under nitrogen flow, resuspended in 200 μL n-hexane and analysed via GC.

2.9.3: Imine reduction by recombinant enoate reductases in biphasic system water/organic phase

Whole cells of *E.coli* BL21 (DE3) or *E.coli* JM109 (DE3) expressing recombinant glucose dehydrogenase and enoate reductase from *Clostridium acetobutylicum* (CaERI) as well as the enoate reductase from the metagenome (MERI) were resuspended with deoxygenated potassium/sodium 0.1M phosphate buffer pH 7.0 up to an optical density of about 5.0.

500 μL of cell suspension was added to a GC glass vial containing 500 uL of the same buffer and glucose 40mM, thus achieving a final concentration of 20mM of glucose for the cofactor recycle. The reaction started overlaying the water phase with 1 mL of n-hexane containing 10mM of benzylidenmethylamine. The GC vials were incubated at different temperature and time ranges (tipically 25-37-45°C for 6-18-24-48 hours). After incubation the n-hexane phase was transferred to a new GC vial and analyzed via GC. The pH of the water phase was raised to about 12 with NaOH 1M and extracted twice with an equal amount of ethyl acetate. The reaction products were dried under nitrogen flow, resuspended in 200 μL n-hexane and analyzed via GC.

2.9.4: Cinnamic acid reduction by recombinant enoate reductases

Fresh cell cultures (15 mL) of *E.coli* BL21 (DE3) or *E.coli* JM109 (DE3) producing the recombinant glucose dehydrogenase and the enoate reductase of *Clostridium acetobutylicum* (CaERI) as well as the enoate reductase from the metagenome (MERI) were centrifuged in sterile falcon tubes at 4,600 rpm for 30 minutes at 4°C. After supernatant drain off, the cells were washed with an equal amount of the specific anaerobe sterile buffer and centrifuged again under the same conditions. The obtained pellets were resuspended in 3 mL of the desired sterile anaerobe buffer in falcon tubes to a final optical density of about 5.0. The reaction was started mixing 1 mL of cells suspension with 7 mL of the specific buffer under examination and 1 mL of sterile anaerobe solution 200 mM glucose in deionised water and 1 mL of anaerobic sterile cinnamic stock solution (100mM). The solution was then aliquoted in 2 mL eppendorf tubes and incubated at fixed temperature and time (typically 25-37-45-55°C for 2-5-24 hours).

After the proper incubation time the pH of the reaction's mixture was acidified to about 2 with HCl 1M and extracted twice with an equal amount of ethyl acetate. The products in the organic phase were dried under nitrogen flow; resuspended in 0.5 mL of acetonitrile-water mixture (125 μL acetonitrile and 375 μL water at pH 3.0) and analyzed via HPLC.

2.9.5: Benzaldoxime reduction by recombinant enoate reductases

Fresh cell cultures (15 mL) of *E.coli* BL21 (DE3) or *E.coli* JM109 (DE3) producing the recombinant glucose dehydrogenase and the enoate reductase of *Clostridium acetobutylicum* (CaERI) as well as the enoate reductase from the metagenome (MERI) were centrifuged in sterile falcon tubes at 4,600 rpm for 30 minutes at 4°C. After supernatant drain off, the cells were washed with an equal amount of the specific anaerobe sterile buffer and centrifuged again under the same conditions. The obtained pellets were resuspended in 3 mL of the desired sterile anaerobe buffer in falcon tubes to a final optical density of about 5.0. The reaction was started mixing 500 μL of cells suspension with 500 ul of the sterile specific buffer containing 0.02M benzaldoxime and 0.04M glucose for the cofactor recycling in 2 mL eppendorf tubes. The tubes were incubated at fixed temperature and time (typically 25-37-45°C for 6-18-24-48 hours). After the desired incubation time the eppendorf tubes were centrifuged in a table centrifuge at

14.500 rpm for 5 minutes and the clear supernatant was filter sterilised and analyzed via HPLC.

2.10: Hydrolysis of N-acetyl-Benzylmethylamine

2.10.1: Specific coloration for secondary amines

Recombinant *E. coli* cells DH5alpha carrying empty pUc19 vector were grown at 37°C in deep multiwell plates in the presence of three different amines (benzylmethylamine; benzyl-phenyl-amine; methyl-phenethyl-amine) at different concentrations (0.1%, 0.05% weight per volume) in different media (Luria Bertani, Terrific Broth and minimal medium M9) supplied with antibiotic for selection of recombinant cells. After 24 hour growth an aliquot of 200 μL was transferred into a clean multiwell plate and stained by adding first 25 μL of a solution of 200mM acetaldehyde in DMSO and subsequently 25 μL of a solution of 200mM tetrachloro-p-benzoquinone in DMSO. After 3 minutes incubation at room temperature the wells with lower concentration of secondary amines and the wells of blanks (broth without secondary amines) were clearly distinguishable by optical means.

2.10.2: Hydrolysis of N-Acetyl-benzylmethylamine by lipases in buffer

N-Acetyl-benzylmethylamine was dissolved at concentration of 10mM in 50mM potassium-sodium phosphate buffered alternatively at pH 4.0, 7.0 and 9.0. The reaction was started by adding 1 mL aliquots of the substrate solution in in 2 mL eppendorf tubes containing the lipase under study and incubating the mixtures at the investigated temperature. The reaction products were analyzed after 6-18-24-48-72 hours by TLC using substrates and expected commercial products compounds as reference, with ethyl acetate:diethyl ether 9:1 as mobile phase.

2.10.3: Hydrolysis of N-Acetyl-benzylmethilamine by lipases in organic solvent

N-Acetyl-benzylmethylamine was dissolved in n-hexane saturated with water at the concentration of 0.01M. The reaction started distributing the substrate solution in GC vials containing the lipase under study and incubating the mixtures at the desired temperatures. The reaction products were analyzed after 6-18-24-48-72 hours by TLC using substrates and expected commercial products compounds as reference, with ethyl acetate:diethyl ether 9:1 as mobile phase.

2.10.4: Hydrolysis of N-Acetyl-benzylmethylamine by lipases in biphasic system

1 mL aliquots of the investigated buffer was distributed in different GC vials containing the lipase under study. The reaction was started overlaying the water phase with 1 mL of n-hexane in which N-acetyl-benzylidenamine had been dissolved at concentration of 10 mM. The reaction products were analyzed after 6-18-24-48-72 hours by TLC using substrates and expected commercial products compounds as reference, with ethyl acetate:diethyl ether 9:1 as mobile phase. Both the organic phase and water phase were separatedly spotted onto TLC plates for content analysis.

2.10.5: Hydrolysis of N-Acetyl-benzylmethylamine by proteases in water phase

N-Acetyl-benzylmethylamine was dissolved at concentration of 0.01M in 0.05M potassium sodium phosphate alternatively at pH 4.0, 7.0, 9.0. The reaction was started by adding 1 mL aliquots of the substrate solution in 2 mL eppendorf tubes containing the protease under study and incubating the mixture at the investigated temperature. In the case of papayn, mercaptoethanol was added to the buffer in the final concentration of 1mM as described in literature (Wang, 1995) in the investigated buffer (potassium-sodium phosphate 0.05M pH 4.0-7.0-9.0) at the concentration of 0.01M. The reaction started aliquoting 1 mL of the investigated buffer to a 2 mL eppendorf tube containing the investigated protease. The reactions were analyzed after 6-18-24-48-72 hours by TLC using substrates and expected commercial products compounds as reference, with ethyl acetate:diethyl ether 9:1 as mobile phase. Both the organic phase and water phase were separately spotted onto TLC plates for content analysis.

2.11: Analytical techniques

2.11.1: HPLC analysis

The conversion of caffeic acid to hydroxycaffeic acid by microbial or enzymatic reduction was analyzed with HPLC using a 100/5C18 column (250 x 4 mm) and a UV detector 166 (Beckmann Coulter). The run was performed with a gradient of the mobil phases water and acetonitrile, it started with 5 minutes at 90% water, later water to 0% in 20 minutes, later 5 minutes with 0% water, later to 90% water in 5 minutes and final hold of other 5 minutes, overall time 40 minutes. The water was acidified to pH 3 with phosphoric acid 84% HPLC grade, the 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.

The retention time for the substrate caffeic acid was 17.6 minutes, the retention time of the dihydroxycaffeic acid (the product) was 16.5 minutes.

The conversion of cinnamic acid to hydroxycinnamic acid by microbial or enzymatic reduction was analyzed with HPLC using a 100/5C18 column (250 x 4 mm) and a UV detector 166 (Beckmann Coulter). The run was performed with a gradient of the mobil phases water and acetonitrile The run was performed with a gradient of the mobil phases water and acetonitrile, it started with 5 minutes at 90% water, later water to 0% in 30 minutes later 5 minutes with 0% water and later to 90% water in 5 minutes, overall time 45 minutes. The water was acidified to pH 3 with phosphoric acid 84% HPLC grade, the 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.
The retention time for the substrate cinnamic acid was 14.2 minutes, the retention time for the dihydroxycinnamic acid (the product) was 15.0 minutes.

The conversion of benzaldoxime to hydroxybenzaldoxime by microbial or enzymatic reduction were analyzed with HPLC using a 100/5C18 column (250 x 4 mm) and a UV detector 166 (Beckmann Coulter). The run was performed with a gradient of the mobil phases water and acetonitrile The run was performed with a gradient of the mobil phases water and acetonitrile, it started with 5 minutes at 90% water, later water to 0% in 30 minutes later 5 minutes with 0% water and later to 90% water in 5 minutes, overall time 45 minutes. The water was acidified to pH 3 with phosphoric acid 84% HPLC grade, the 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.
The retention time for the substrate benzaldoxime was 28.2 minutes, the retention time for the hydroxybenzaldoxime (the product) was 24.1 minutes.

2.11.2: GC analysis

The conversion of the substrate acetophenone 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 were: hexane 1.2-1.5 minutes, acetone 1.6 minutes, 2-propanol 2.2 minutes, decane 2.95 minutes, acetophenone 10.1 minutes and phenylethanol 11.0 minutes.

The conversion of the substrate benzylidenmethylamine to product benzylmethylamine 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 developed by us to separate also the degradation products (benzaldehyde and benzylalcohol) of the substrate benzylmethylamine.

Original oven temperature: 80 °C for 2 minutes

Temperature increase: 10 °C per minute till 200 °C

End temperature: 200 °C for 1 minutes

Total time duration: 15 minutes

The retention times were hexane 1.2-1.5 minutes, decane 3.2 minutes, benzylidenmethylamine 10.1 minutes, benzaldehyde 10.5 minutes, benzylmethylamine 11.0 minutes, benzylalcohol 11.4 minutes.

2.11.3: 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^{TM} 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^{TM} Protein Staining Solution (MBI Fermentas, Germany) 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		Stacking gel (5%)
	12 %	7.5%	
40% Acrylamide mix	3.0 mL	1.9 mL	0.5 mL
1.5 M Tris-HCl, pH 8.8	2.5 mL	2.5 mL	-
1 M Tris-HCl, pH 6.8	-	-	0.5 mL
10% (w/v) SDS	0.1 mL	-	0.04 mL
10% (w/v) APS	0.1 mL	0.1 mL	0.04 mL
TEMED	0.004 mL	0.004 mL	0.004 mL
Deionized H_2O	4.3 mL	5.5 mL	2.9 mL
Total volume	10 mL	10 mL	4 mL

SDS running buffer:

Tris 3 g/L

Glycine 14.4 g/L

SDS 1 g/L

2.11.4: 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, 1972; Helling, 1974; Wink, 2006) 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^{TM} 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 “QIAGEN, Gel-extraction kit” (QIAGEN, Germany).

50 x TAE buffer, pH 8 (1 liter) 6 x DNA-loading buffer

Tris base 242 g Tris-HCl (pH 7.6) 10 mM

Glacial acetic acid 57.1 ml Glycerol 60% (v/v)

EDTA 18.6 g EDTA 60 mM

Deionized water up to 1 l Bromophenol blue 0.03% (w/v)

CHAPTER 3: RESULTS AND DISCUSSION

3.1: Introduction

The present study is focused on the investigation of possible biocatalytic routes for the reduction of the carbon nitrogen double bond in imines and oximes to secondary amines and hydroxyamines. This kind of reduction is absent from the common enzymatic reactions available in shelves of laboratory as well as in industry.

At the moment no enzymes or bacteria have been described as specific catalysts for this reaction on those substrates, thus it has been decided to investigate the reduction of carbon nitrogen double bonds as promiscuous activity of already known enzymatic systems.

The starting point of this work was the hypothesis that enoate reductases and carbonyl reductases could reduce imines or oximes because of electronic and steric similarities of the class of natural substrates and the substrates under investigation.

The enoate reductases applied in the reactions have been isolated from strains and metagenome; the carbonyl reductase, was available in our laboratory (Dr. Bhattacharjee, PhD thesis, 2006).

Two different methods have been developed *ex novo* for the detection of the desired compound in the enzymatic reaction mixtures and both were compatible with High Throughput Screening assay.

The first was a colorimetric method selective for the detection of the eventually formed secondary amines (other aminic groups present in the reaction media do not interfere); the second one was a UV detection of the residual absorption of the substrate applied in the enzymatic conversion.

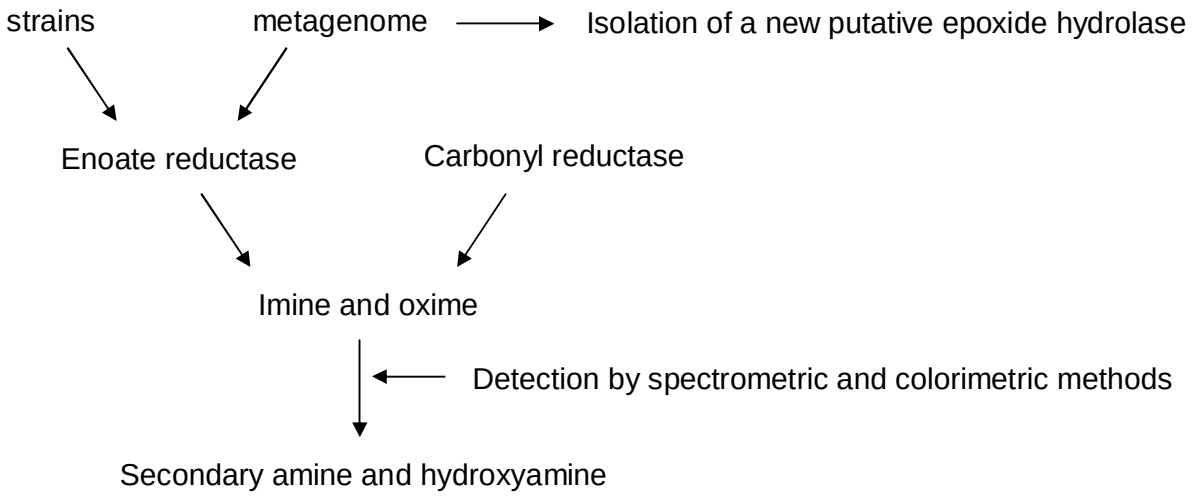


Figure 3.1: Overall description of the project

3.2: Reduction of caffeic acid using *Acetobacterium woodii*

Following the experimental procedure described in section 2.4, a library of *Acetobacterium woodii* was screened for the reduction of caffeic acid.

A library of ca. 9.000 clones was produced. The quality of the library, in terms of average insert size and the frequency of clones harboring *Acetobacterium woodii* DNA within each library, was determined by restriction analysis of 24 plasmids isolated from randomly chosen clones. The restriction analysis indicated that 70% of the selected clones carried an insert and the insert size was ca. 9 kb. This means that ca. 57 Mb of *Acetobacterium woodii* DNA were inserted in *E.coli* and subjected to further screening for the caffeic acid reduction activity in High Throughput format. However no positive clone was isolated.

One possible explanation for the lack of positive hits could be the sensitivity and instability of enoate reductases in the presence of molecular oxygen (Snape, 1997).

Moreover the achievement of full anaerobic conditions in microtiter plates is a technical challenge. In the present study microtiter plates sealed with impermeable rubber lids were used; the *E.coli* cells grew inside the deep well plates first aerobically until all the oxygen was consumed. Thus, the cells as such produced the needed anaerobic environment.

Besides, until today in literature no enoate reductases have been isolated *via* activity based screening of library in *E.coli*, thus leading to the conclusion that *E.coli* might not be the right host for the isolation of these enzymes, neither using TB medium for the searched protein expression. In fact the only paper available at that time (Mueller, 2001) about

expression of enoate reductase in *E.coli* showed that the protein was active only if expressed anaerobically in TB. Therefore, the negative results are consistent with those previous published data.

The choice to use TB medium, rather than LB one, was done for several reasons: as first the LB medium does not contain a buffer system, whereas TB medium is based on a phosphate buffer system, useful for buffering the organic acids produced when *E.coli* is grown anaerobically. In a non-buffered system these acids lower the medium pH, thus inhibiting further cell growth. Moreover the TB is a richer medium, leading to higher biomass production, and thus to a greater theoretical level of expression of the desired protein.

Very recently – when the herein reported screening was concluded –, a new article was published (Imkamp, 2007), in which a deeper study of the *Acetobacterium woodii* caffeic acid reduction mechanism was performed. Actually, this reaction is part of a complex multienzymatic step. The caffeic acid reduction happens in the so called “caffeic respiration” and is a chemiosmotic mechanism with sodium ions as coupling ions, where the caffeate is reduced with the electrons derived from the hydrogen to the synthesis of ATP. Very importantly, caffeic acid would not be the actual substrate of the enzyme, but its activated form, as CoA ester. The fact is rather important for a preliminary HTS screening aiming to identify active clons: it may be possible that enzymes are successfully cloned, but that due to thermodynamic reasons – insufficient substrate activation, acid *vs*. CoA acid –, the reaction cannot be performed. The mechanism proposed by this research group is depicted in figure 3.2.

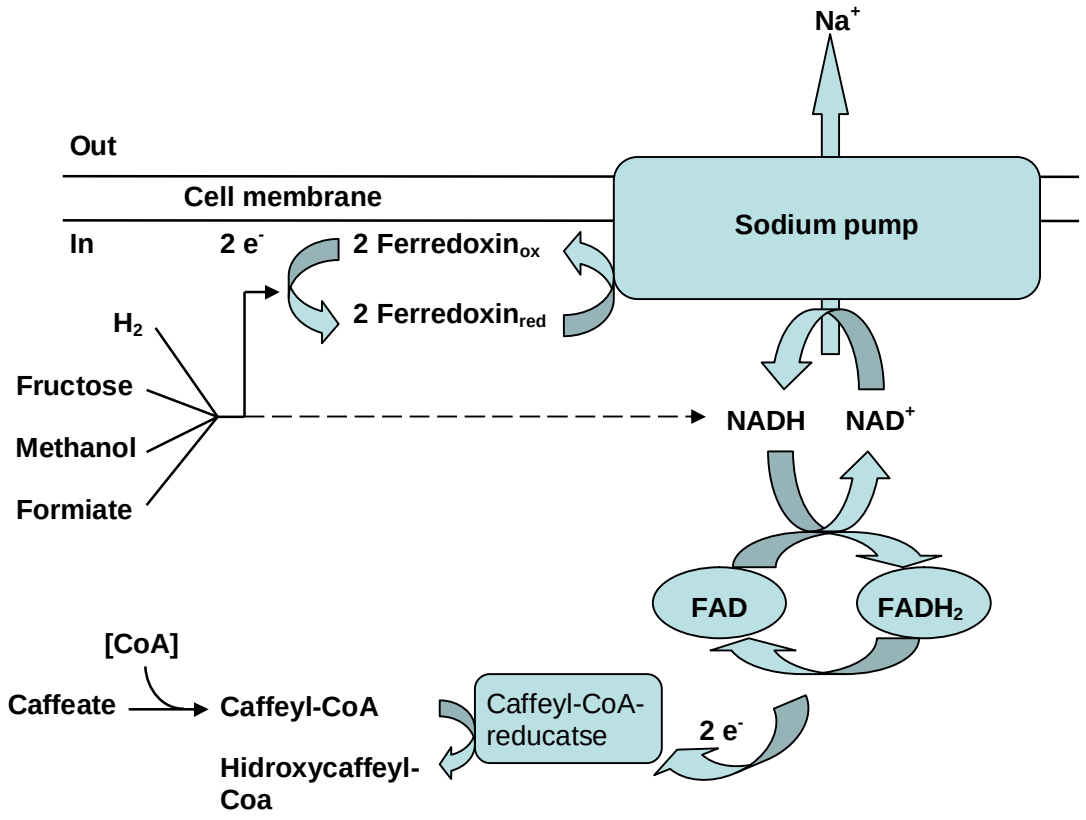


Figure 3.2: Postulated electron flow from various donors to the terminal acceptor caffeate as proposed by Imkamp. Abbreviations: $FADH_2$, reduced form of flavin adenine dinucleotide; FAD, oxidized form of flavin adenine dinucleotide; NADH, reduced form of β-nicotinamide adenine dinucleotide; NAD^+, oxidized form of Noteworthy, it can be noticed that caffeic acid is not reduced as free substrate, but as ester with Coenzyme A.

In order to overcome the challenges of low protein expression in the library and sensitivity toward molecular oxygen another approach has been tried.

A sequence based screening has been performed, involving PCR reactions with the use of degenerate primers to amplify the conserved regions of enoate reductases.

Two sets of primers have been designed, based on the alignments of already reported enoate reductase. The primers have been tried with the genomic DNA of *Acetobacterium woodii* and *Sporomusa termitida* as template in PCR reactions. Both these strains are well known in literature for reducing caffeic acid (Lenourry, 2005; Li, 2004).

However, no amplification products from the PCR reactions were observed.

The lack of amplification with these mentioned PCR reactions was assumed as indirect proof that the enzymes responsible for the caffeic acid reductase activity in both the strains were not real enoate reductases (E.C. 1.3.1.31), but more probably belonged to the

dienoyl-CoenzymeA reductase family (E.C. 1.3.1.34) as suggested later by Imkamp (Imkamp 2007) (see above).

It must be reminded at this point that the original hypothesis of the screening of the genomic library was that *Acetobacterium woodii* could show the reduction of enoate reductatse as promiscuous activity of a single isolable enzyme.

As the screening target was the isolation of an enzyme able to (promiscuously) reduce imines to the corresponding secondary amines, an alternative screening method in High throughput format was designed *ex novo* and developed, as shown in paragraph 2.10.1.

Nevertheless, the decision of screening the genomic library of *Acetobacterium woodii* targeting caffeic acid reduction, instead of secondary amines formation, was made for several reasons.

As first the ability of enzymatic C=C reduction was postulated in the work of the research group of G. Stephens (Li, 2004.) as possible candidate for the promiscuous reduction of C=N reduction. Secondarily caffeic acid is a cheap substrate and easy to handle and detect: it shows a strong absorbance at 310 nm, allowing the detection at low concentrations (0.8 10^{-3}M substrate concentration in the performed screening). A third and even maybe more important reason to screen for caffeic acid, is intrinsic to the "promiscuity concept". When an enzyme in nature shows a promiscuous activity, or when it is artificially evolved toward that goal, usually the activity for the "promiscuous" substrate shows a significantly lower turnover number than the activity for his natural class of substrates, even some magnitude orders lower. Therefore, since the expected conversions could not be very high, the sensitivity of the screening technique plays in this case a crucial role.

Considering all these reasons, and especially the sensitivity of the screening for caffeic acid reduction in High Throughput screening, the decision of screening the *Acetobacterium woodii* library in multiwell plates for the substrate caffeic acid instead of the product secondary amines was made, even if a screening for the secondary amines appearance was already developed at the beginning of this study (see section 3.5).

Besides, the assay developed in High Throughput format for the detection of secondary amines was not used to screen the library also because it involved a two step dyeing reaction and moreover the promiscuous substrate (the imine) showed instability in water, requiring the need to use a second organic phase, thus making the whole process of product detection cumbersome, when compared to the simple UV measurement of caffeic acid in TB medium, as in scheme 3.2.

Caffeic acid method	Tetrachlorobenzoquinone method
cultivation of E.coli cells in deep well plates with substrate ↓ **deep well plates centrifugation** ↓ **transfer of aliquotes in multiwell plates** ↓ **UV measurement at 310 nm to quantified the residual caffeic acid**	**cultivation of E.coli cells in deep well plates supplied with imines** ↓ **transfer of aliquotes of growth medium in multiwell plates** ↓ **addition of acetaldehyde** ↓ **addition of tetrachlorobenzoquinone** ↓ **optical detection of color formation**

Scheme 3.2: Comparison of the two High Throughput Screening techniques developed in this study.

3.3: Reduction of caffeic acid using a metagenomic library

The choice of the biological sample, when a metagenomic screening is performed, represents a crucial step.

In this study, the goal of the metagenomic screening was the isolation of a enoate reductase enzyme. The enoate reductase belongs usually to anaerobic bacteria, thus the decision of mining an anaerobic environment has been made.

In an ideal gene library with equal representation of all indigenous species and no non-productive clones, the number of clones that statistically need to be screened to find a positive one is solely determined by the frequency of organisms carrying one or several genes of interest in the source DNA. This frequency can be raised by a classical enrichment step (Gabor, 2004) preceding DNA isolation, where organisms are cultivated under selective pressure that favors the growth of bacteria expressing a desired activity. Like traditional strategies for enzyme discovery, this method may suffer, of course, from the fact that many organisms will not grow under laboratory conditions due to their special requirements in (nutritional) growth conditions. Additionally, many so-called “non-

cultivable" bacteria, cannot be obtained as pure isolates, since they exist in nature as symbionts or as part of consortia, requiring the presence of certain other organisms for growth.

Based on the method described in section 2.3.3 the metagenomic DNA was isolated from enriched environmental samples.

In order to enhance the overall DNA yield, a modification of the Zhou protocol was performed. The modification consisted in freezing the environmental sample with liquid nitrogen and later grinding it in a sterilized mortar, as described in the section 2.3.3 of materials and methods.

The effect of this modification to the normal protocol is clearly visible in figure 3.3: on the left side there are DNA samples obtained with the modified protocol, instead on the right side there are DNA samples obtained from different aliquotes of the same environmental enriched sample, but extracted with the standard Zhou method as control.

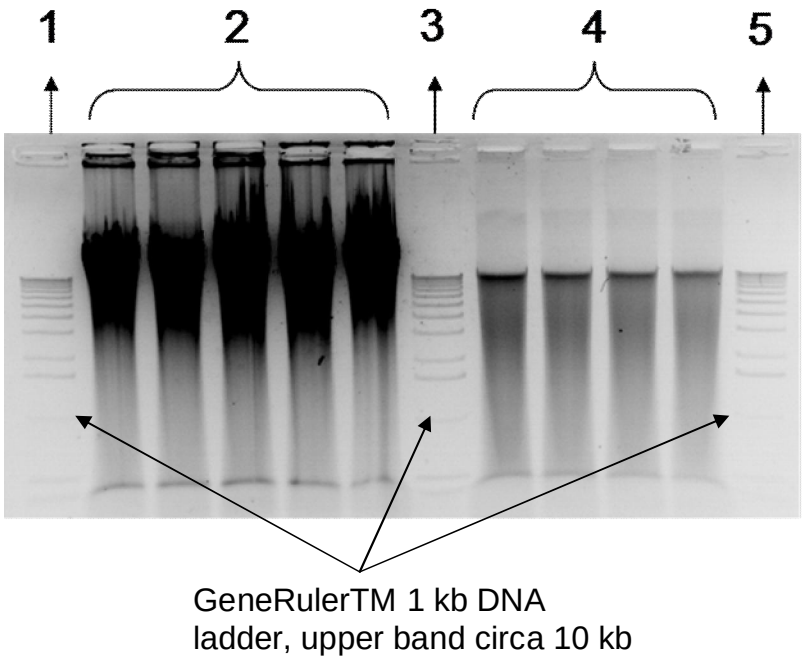


Figure 3.3: The effect of grinding the environmental sample under liquid nitrogen.
Line 1; 3 and 5: GeneRuler™ 1 kb DNA ladder, upper band circa 10 kb. Slots number 2: 5 µL of metagenomic DNA isolated coupling Zhou extraction method and grinding the frozen samples in a mortar with liquid nitrogen.
Slots number 4: 5 µL of metagenomic DNA isolated with the standard Zhou method.

The improvement in the overall yield due to the grinding with liquid nitrogen was estimated of being at least 10 times higher.

The obtained DNA was cloned and screened in *E.coli* cells as described in section 2.4.5 and 2.6

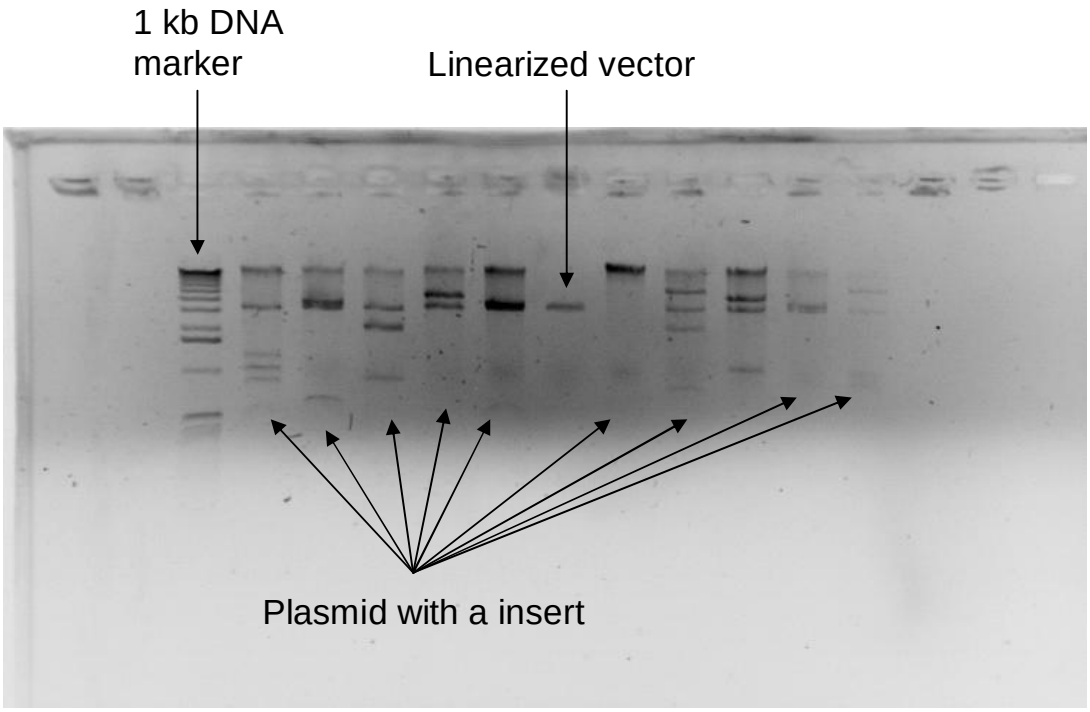


Figure 3.4: Plasmid analysis of the library constructed with metagenomic DNA. As shown by the arrows virtually all the analyzed clones have plasmids with an insert; the plasmids have been digested with two restriction enzymes flanquing the multiple cloning site. The linearized vector is added as control.

The quality of the obtained library was evaluated as described in section 2.4.6. The results of the plasmid analysis are shown in figure 3.4.

However, from this screening, no conversion of caffeic acid was detected.

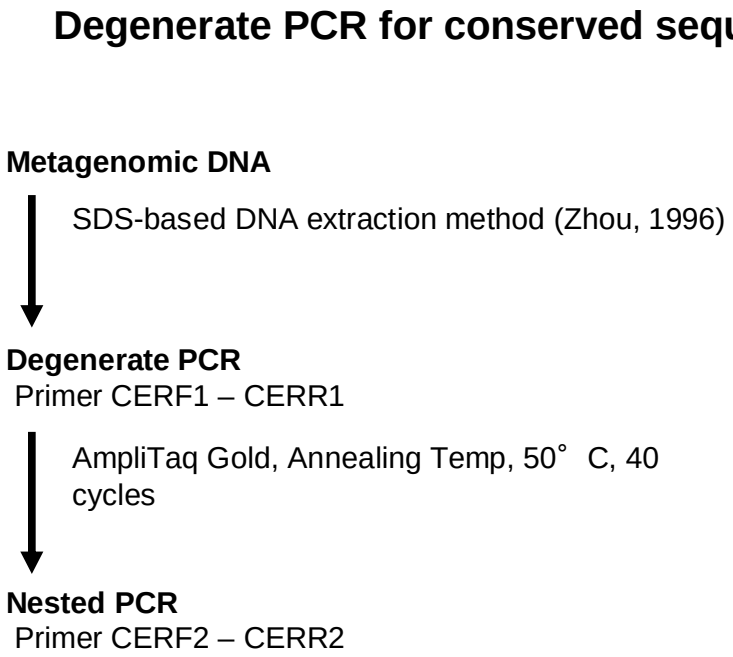
As already mentioned above, several reasons can be postulated to justify the fact that no positive clone for the reduction of caffeic acid was found in the activity screening.

Among the reasons that could be mentioned once again the impossibility to reach full anaerobic conditions within the microtiter plates could have played a major role. In addition, the lack in literature of enoate reductases retrieved by genomic cloning and functional analysis led to conclusion that the strategy chosen was not fitting with the goal.

The used vector p-Zero-2 was chosen for several reasons. As first the vector has a smaller size (3,3 kb), compared to the PWE15 (8.1 kb) vector applied for the construction of the *Acetobacterium woodii* library, thus allowing a higher transformation efficiency (in respect of number of clones per cloned DNA). Secondarily, it is a vector that positively select transformants with a insert, because in the case of self-religation the clones express the CCDB suicide protein for *E. coli* and then the clones that harbour the self-religated vectors are not able to grow (p-Zero-2 cloning manual), enhancing the quality of the library.
The size of the obtained library was ca. 20.000 clones with an average insert size of circa 5 kb, virtually free of religants. In any case the assumption that 5% of the clones were anyway religants was made, according with manufacturer suggestions, even if the plasmid analysis in figure 3.3 showed that no religants were indeed present.
This means a screen of circa 95 Megabases of uncharacterized DNA.

3.4: Isolation of a new enoate reductase from the Metagenome

The metagenomic DNA obtained as descriebd in section 2.3.3 was used also as template for PCR amplifications with degenerate primers as described in section 2.4.3 and resumed in scheme 3.3.



Scheme 3.3: Strategy followed to isolate enoate reductases from the metagenome.

The results of the amplification led to the isolation of a new enoate reductase. The procedure used for the isolation of the complete DNA fragment encoding for the enzyme was performed following a published work (Uchiyama, 2006), an adaptation of the Genome Walker™ (Clontech, USA) and resumed in figure 3.5.

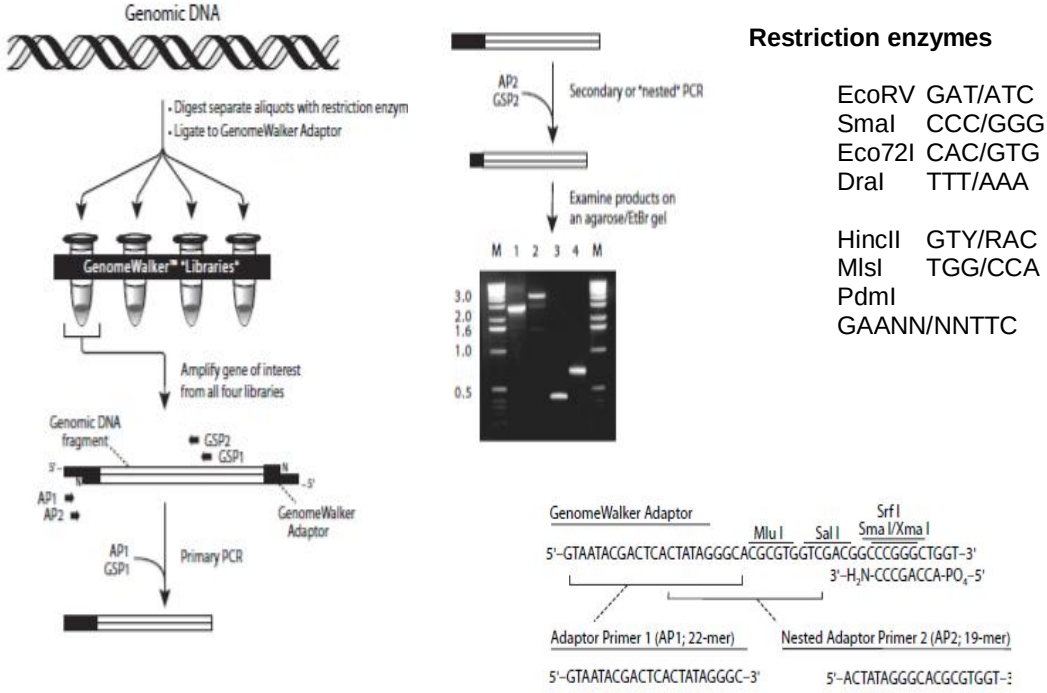


Figure 3.5: Strategy followed to isolate the rest of the gene encoding for the new enoate reductase isolated from the metagenome

Several DNA templates were tried in parallel for the primary PCR amplifications; among them also the gDNA of *Acetobacetrium woodii* and the gDNA of *Sporomusa termitida.*

The degenerate primers used for the PCR amplifications, were based on the alignments of three different enoate reductases, as shown in figure 3.6.

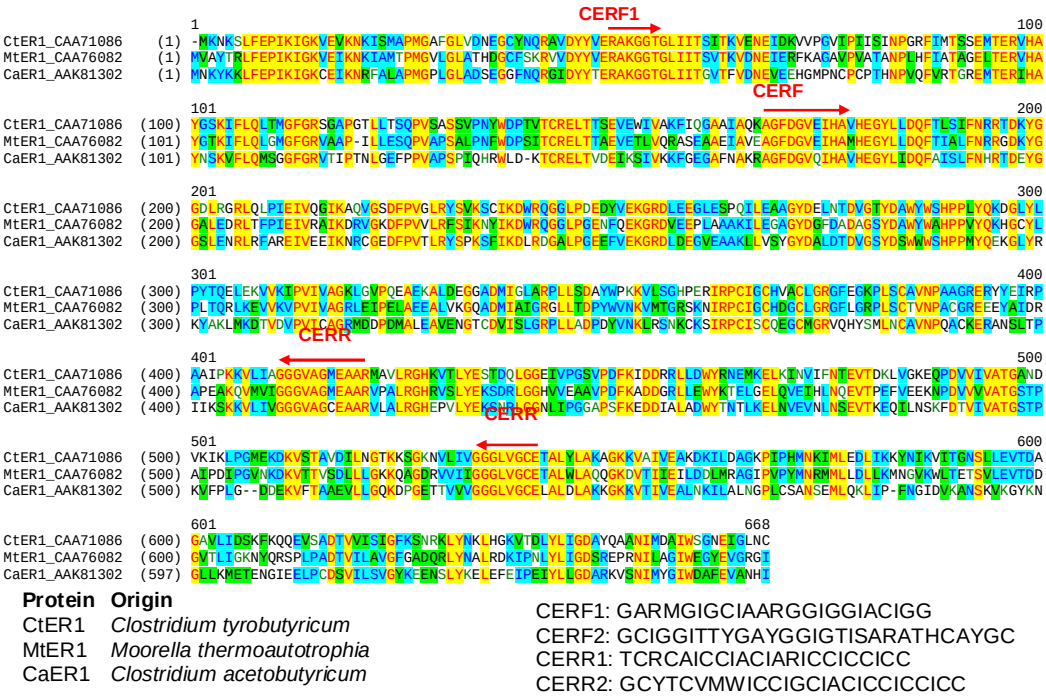


Figure 3.6: design of the degenerate primers used in this study, based on the alignment of already known enoate reductases.

Among the different DNA sources only the metagenomic DNA of the enrichment gave an amplification product from the primary PCR. The gDNA of the two strains *Acetobacterium woodii* and *Sporomusa termitida* instead didn't give any amplification products, thus leading us to the conclusion that the caffeic acid reductase in both strains are not related with the ones used in the alignment.

The alignment of the obtained gene of the enoate reductase retrieved from the metagenome with the other three enoate reductases used to design the degenerated primers is shown in figure 3.7.

The optimization of the co-expression of this new enoate reductase together with a glucose dehydrogenase to recycle the cofactor is discussed in sections 2.9.1 and 3.7.1.

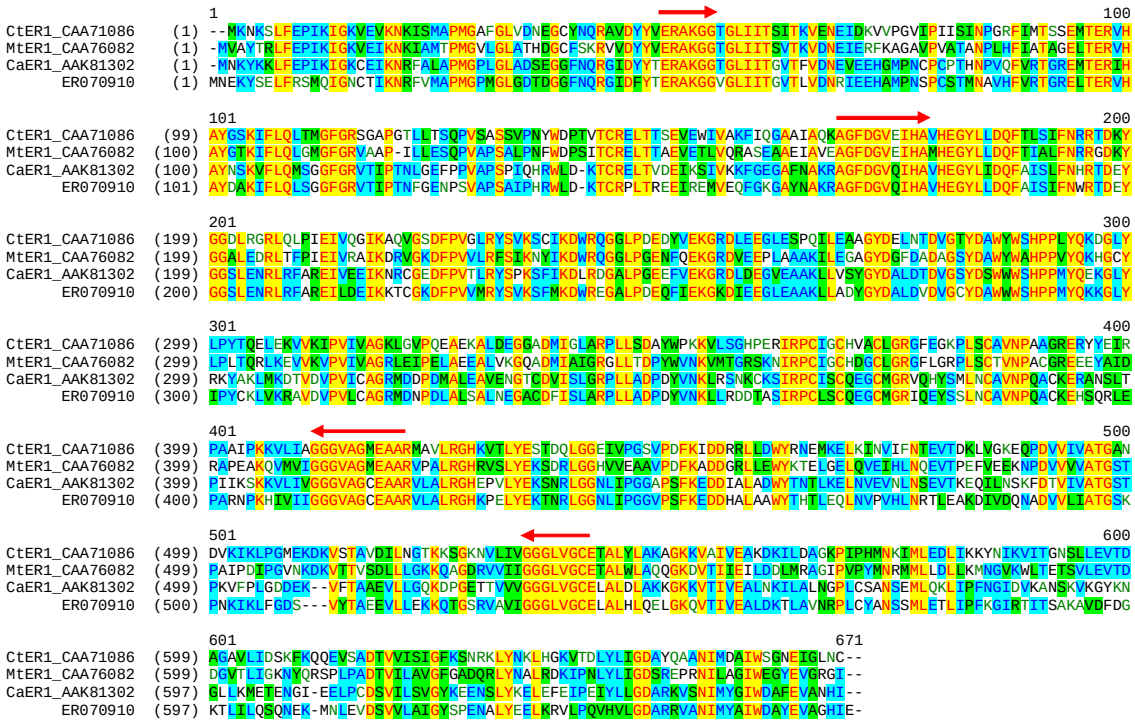


Figure 3.7: The alignment of the new enoate reductase isolated from the metagenome following the protocol of Uchiyama (Uchiyama, 2006) with the three enoate reductases aligned and used to degenerate the primers.

3.5: Development of a selective screening to target secondary amines

An alternative screening technique was developed, allowing the detection of the presence of secondary amines in the cultivation broth (LB or M9 mineral media) in High Throughput format, see section 2.10.1.

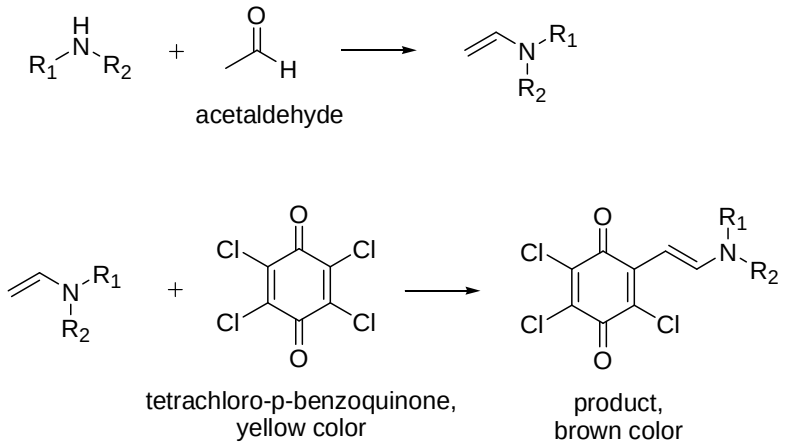


Figure 3.8: Description of the two steps derivatization of secondary amines in the developed test.

A selective coloration for secondary amines was adapted from a qualitative assay from solid-phase chemistry (Voikovsky, 1995)

Secondary amines are the product of the bioreduction of imines and they could be detected with a two steps derivatization reaction (Figure 3.8).

As first, acetaldehyde was added to the secondary amine; the reaction between aldehyde and amino groups was fast. The formed condensation compound reacted with a solution of Tetrachloro-p-benzoquinone, added in a second step, leading to a dark greenish-reddish product. The color formation happened within the first 5 minutes since the addition of the quinone solution. Using a calibration curve with different amount of secondary amine; it has been demonstrate the possibility of a semiquantitative detection by optical means that allowed to distinguish among 0%-25%-50%-75%-100% of secondary amine added in the medium; the coloration was stable up to 4-6 hours (figure 3.9).

The coloration was selective for secondary amines (the presence of primary and tertiary amines led to a distinctly different coloration (data not shown).

Those latter are only formed with secondary amines) and thus in principle it gave very accurate results. Only at prolonged incubation times a background coloration appeared even in the blanks. This was probably due to primary amines present in the media (free amino acids, ammonia salts, etc.) reacting with acetaldehyde to an imine intermediate, which as a consequence of low spontaneous interconversion to enamine reacted with Tetrachloro-p-benzoquinone giving the colored reaction product, as cleared in figure 3.10.

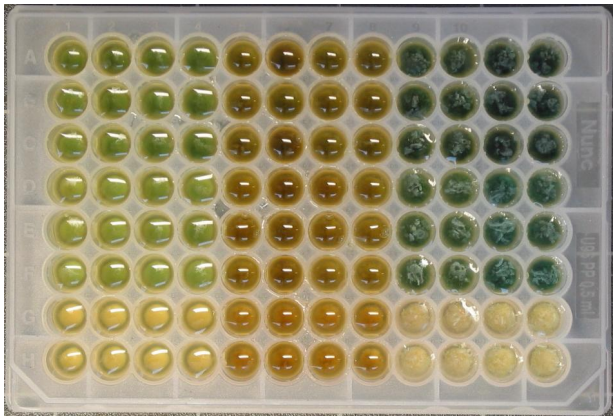


Figure 3.9 Microtiter plate with stained broth. Rows 1-4: LB medium; rows 5-8: TB medium; rows 9-12: M9 medium; lines A-C: N-methyl-1-phenylmethanamine 0.1% w/vol; lines D-F: N-methyl-1-phenylmethanamine 0.05% w/vol; lines G-H: blank.

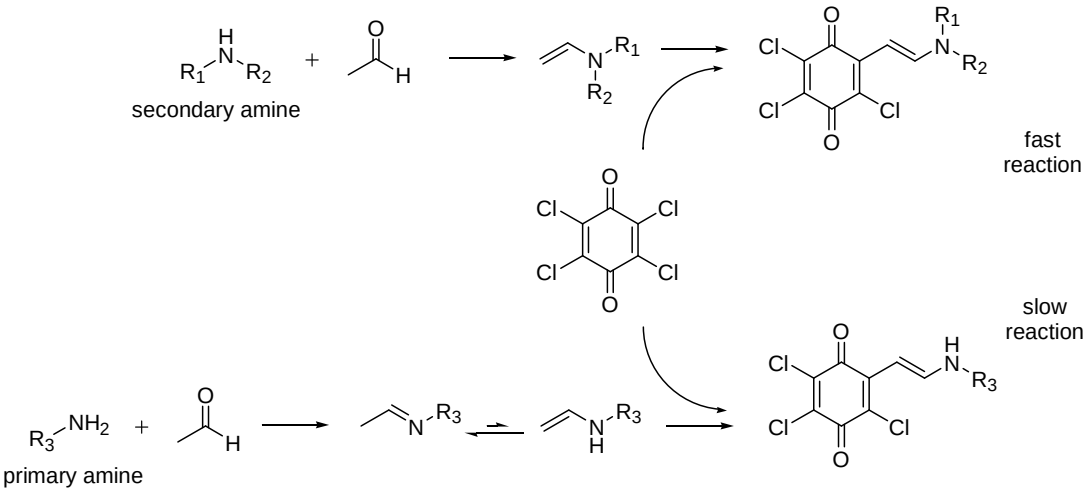


Figure 3.10: Coloration of the secondary amines (fast reaction) and of primary amines (slow reaction); the last occurred only on prolonged incubation time.

3.6: Hydrolysis attempts of N-Benzyl-N-methylacetamide

Since an enzymatic reaction able to produce secondary amine was not present in our laboratory, it has been decided to investigate the preparation of this secondary amine anyway biocataliyically. The aim was the use of the possible hydrolytic reaction as positive control for the selective screening developed to target secondary amines aa described above.

As shown in figure 3.11 the secondary amine could be biocatalytically obtained either from the reduction of the iminic bond of the corresponding imine, or from the hydrolysis of the N-aceto ester

N-Benzyl-N-methylacetamide was synthetized, as described in section 2.1.1, to use it as substrate in the developed screening for secondary amines.

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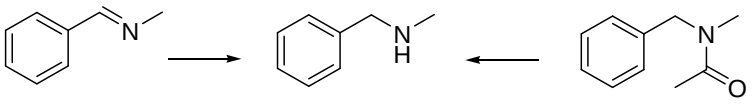


Figure 3.11: Possible biocatalytic routes for the production of the secondary amine benzylmethylamine.

As several hydrolases, namely lipases and proteases (Bornscheuer, 2005), are reported in literature for the ability of promiscuously hydrolyzing different N-acetamidic bonds, a screening has been performed in order to find an enzyme able to hydrolyze specifically N-Benzyl-N-methylacetamide and release N-Benzyl-methylamine. The main challenge of this screening consisted in the selected substrate: a survey of the current literature showed that primary amides are the described substrates in the above mentioned reactions, instead the selected substrate is a secondary amide.

The enzyme preparations of the “lipase and esterase screening kit” from Sigma-aldrich were tried. They consisted of 18 different hydrolytic enzymes (listed in table 3.1); each of them has been tried in pure organic solvent, or in buffer at pH 4.0, 7.0, 9.0, and in biphasic system with the water phase at pH 4.0, 7.0, 9.0. The solvent of choice was n-hexane in all the tested reactions and the substrate concentration was always 0.01M.

With any tested enzyme no hydrolysis has been observed, even prolonging the reaction time to some days and analyzing the reaction mixture via TLC.

The hydrolysis of the substrate has been tried also with 5 different commercial proteases under different combinations of pH and temperature and even prolonging the reaction time to some days, but no product formation was detected *via* TLC.

In conclusion none among the tested hydrolytic enzymes showed activity toward the hydrolysis of N-Benzyl-N-methylacetamide, thus the screening developed for the detection of secondary amines formation in High Throughput screening lacks until now a positive control (Section 2.10.1).

Table 3.2: list of the enzymes tested for the hydrolysis of N-Benzyl-N-methylacetamide was synthetized

Lipases	**Proteases**
Aspergillus	Papain
Aspergillus oryzae	*Aspergillus oryzae*
Candida antarctica	*Bacillus sp.*
Candida cylindracea	Chymotrypsine
Candida lipolytica	Bacillus polymyxa
Chromobacterium viscosum	Porcine Kidney
Mucor javanicus	Penicillinase
Mucor miehei	trypsine
Pseudomonas cepacia	
Pseudomonas fluorescens	
Rhizopus arrhizus	
Rhizopus niveus	
hog pancreas	
Pseudomonas fluorescens	
Pseudomonas sp.	
Penicillium roqueforti	
wheat germ	

3.7: Application of enoate reductase for the promiscuous reduction of carbon nitrogen double bond

Expression of enoate reductases

Four different plasmids (table 3.2) with the insertion of the enoate reductse genes in pET22b+ vector were transformed in *E.coli* cells JM109 (DE3) and BL21 (DE3) strains and checked for activity.

In these four plasmids two different enoate reuctases were cloned, one from *Clostridium acetobutylicum*, expressing an enzyme already described in literature (Rohdich, 2001), and the other gene was isolated in the metagenomic screening, as described in section 2.4.7.

Table 3.2: plasmids used within the preliminary expression experiments.

Plasmid name	enzyme	With 6HisTag on N-Terminus
pCaERI	*Clostridium acetobutylicum* Enoate reductase	No
pCaERIHisTag	*Clostridium acetobutylicum* Enoate reductase	Yes
pMER	Metagenome isolated Enoate reductase	No
pMERHisTag	Metagenome isolated Enoate reductase	Yes

The fact that the cells had to be induced, harvested, washed and prepared as biocatalyst under strict anaerobic conditions made all the experiments time consuming and cumbersome compared to other class of enzymes, then all these steps were performed in a house-built anaerobic chamber (figure 3.12).

Figure 3.12: The house-built anaerobic bench, where all the handling and preparation steps of the biomass containing the enoate reductase enzymes were carried.

To verify the correct expression of the enoate reductase in the cells, the reduction of cinnamic acid to the corresponding 3-phenylpropionic acid (figure 3.13) was tested using the whole cells as biocatalyst. This compound has been chosen because it is the natural substrate for these investigated enzymes.

In order to find with a rational approach the best conditions for the induction of the desired activity, as first a small experimental design was set, varying the induction conditions as indicated in table 3.3, and starting the induction at an optical density of 0.5.

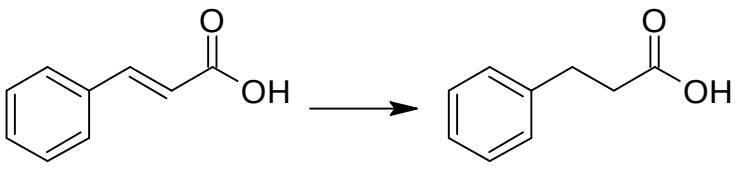


Figure 3.13: The activity test reaction in the expression experiments for the different enoate reductases. The double bond of cinnamic acid (left side) is reduced by the enoate reductase, obtaining 3-phenylpropionic acid.

Table 3.3: the experimental conditions tried at the beginning of the optimization process; IPTG concentration is expressed in mM; Time in hours and Temperature in °C.

Experiment n.	IPTG	Time (h)	Temperature
1	0.2	3	25˚C
2	0.2	6	30˚C
3	0.2	9	37˚C
4	0.6	3	30˚C
5	0.6	6	37˚C
6	0.6	9	25˚C
7	1	3	37˚C
8	1	6	25˚C
9	1	9	30˚C
10	0.6	6	30˚C
11	0.6	6	30˚C
12	0.6	6	30˚C

During these prelilminary activity tests it has been demonstrated that the cells expressing the enoate reductases without the 6HisTag at the N-terminus of the protein showed activity, while the ones with the 6HisTag were not active. Besides, between the two *E.coli* strains, JM109 (DE3) showed slightly higher activity compared to BL21 (DE3). The cells with the native enoate reductase either from *Clostridium acetobutylicum* or from the metagenome showed the highest activity in the experiment number 6, namely with 0,6 mM IPTG, 9 hours of expression at 25 °C.

However, the difference in activity was significantly lower for the cells expressing the plasmid pMER than expressing the plasmid pCaERI.

After the activity test a SDS acrylamide gel was run, in order to check possible over-expressing bands, but no clear bands were visible for both the enzymes (data not shown).

The activity test was performed anaerobically, in 2 mL eppendorf tubes. In the reaction mixture two equivalents of NADH pro equivalent of cinnamic acid was added from a concentrated stock solution (0.02M of NADH for 0.01M of cinnamic acid).

The conversion of cinnamic acid, after overnight incubation at 30 °C, was not complete with any of the 12 experiments in the applied statistical design. The greatest conversion,

as mentioned above, was reached with the experiments number 6, it was 68% for the pCaERI and 45% with the pMER respectively.

In order to handle strains more suitable for routinely investigation in the laboratory and to easily exploit the potential of the enzymes, a cofactor regeneration system was required. To achieve this goal a variant of Glucose dehydrogenase improved by directed evolution in the research group of professor Sarayama (Biotechnology center, Tokyo, Japan) was applied.

The plasmid of the above mentioned enzyme was cotransformed together with the plasmids pCaERI and pMER again in *E.coli* JM109 (DE3). The obtained *E.coli* strains harboured both the enzymes and the optimization of the overall activity was performed once again.

The optimization of the coexpression conditions to obtain a biocatalyst that significantly reduced cinnamic acid revealed to be recalcitrant, and after many different conditions assayed attempts it has been discovered an optimum using conditions as 2,5 10^{-3}M IPTG, 18 hours at 25 °C, starting the induction when the cells reached a optical density of ca. 1.

The figure 3.14 shows bands of the enzyme over-expression after incubation of the *E.coli* cells with the above mentioned conditions.

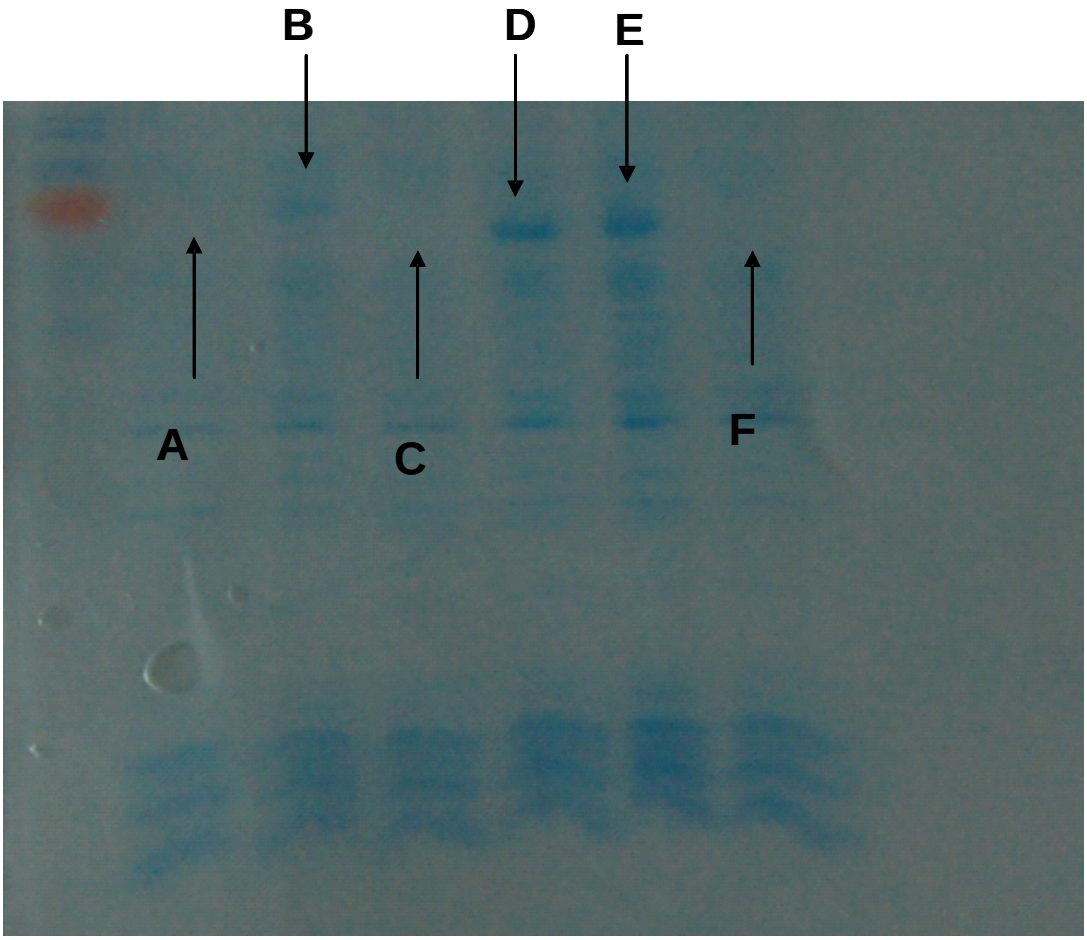


Figure 3.14: SDS-acrilamide gel analysis of the expression of the two enoate reductases induced as mentioned in the text.

Under these conditions the reduction of 0.01M cinnamic acid was achieved within five hours at 37 °C with 5% wet cells E.coli JM109 (DE3) with pCaERI and within 18 hours at 37 °C with pMER.

The optimized expression for both the enzymes was a prerequisite to use them in the promiscuous attempts for the of benzyilidenmethylamine and benzaldoxime bioreduction.

Bioreduction attempts of benzylidenmethylamine with enoate reductases

The two strains of *E.coli* harboring the actively co expressed enoate reductases and the glucose dehydrogenase were used as test system in a serie of bioreduction attempts with benzylidenmethylamine.

It has been believed that benzylidenmethylamine could be a promiscuous substrate for the enoate reductase since it has a similar steric hindrance of the natural substrate cinnamic acid, as shown in figure 3.15.

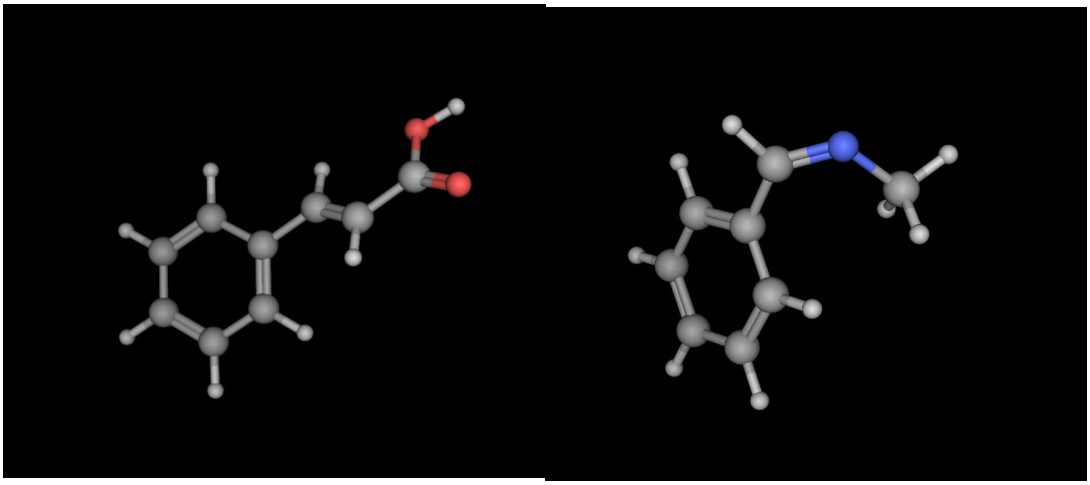


Figure 3.15: Three dimensional representation of the natural (cinamic acid, left side) and promiscuous (Benzylidenmethylamine, right side) substrates for the enoate reductase.

The bioreduction of benzylidenmethylamine has been tried under different combinations of temperature and pH, either in water phase and in biphasic system, as cleared in the table 3.4.

Table 3.4: Resume of the different reaction conditions tried in the bioreduction attempts with the cloned enoate reductases.

Enzyme	**pH**	**Temperature**	**Organic phase**	**conversion**
CaERI	6.0; 7.0; 8.0	30; 37; 45; 55 °C	No	No
CaERI	6.0; 7.0; 8.0	30; 37; 45; 55 °C	Yes	No
MER	6.0; 7.0; 8.0	30; 37; 45; 55 °C	No	No
MER	6.0; 7.0; 8.0	30; 37; 45; 55 °C	Yes	No

The reaction system for the bioreduction attempts is described in details in the section 2.9. The substrate Benzylidenmethylamine turned out to be unstable within the reaction time course. The spontaneous hydrolysis in water of the substrate is resumed in the figure 3.16.

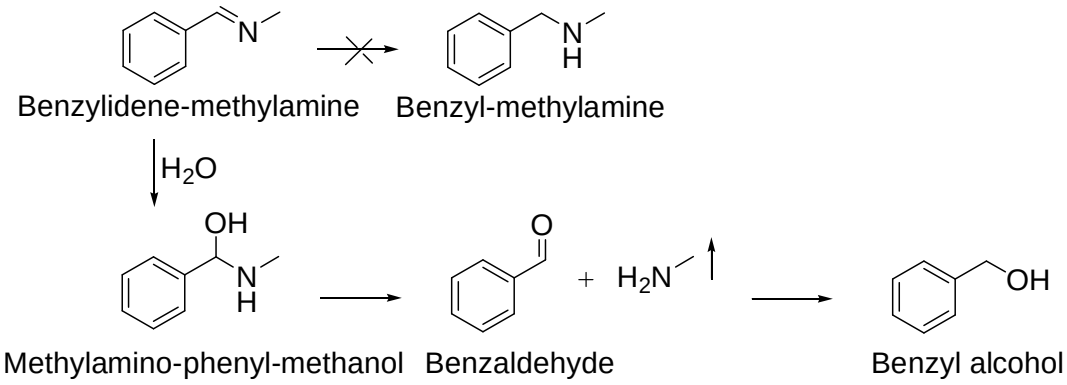


Figure 3.16: the hydrolysis pathway of Benzylidenmethylamine in water phase. In the case of bioreduction attempts with recombinant enoate reductases it was observed a decrease of the substrate concentration within the reaction time and the appereance of benzaldehyde, that later was reduced to benzylalcohol by the constitutive alcohol dehydrogenases present in the *E.coli* strain.

When the bioreduction of benzylidenmethylamine was tried in water phase, GC analysis over the time course of the reaction showed the formation of benzaldehyde within the first 12 hours, followed by decrease of the benzaldehyde concentration and accumulation of benzylalcohol. Sampling the reaction mixture after 24 hours revealed only traces of benzylidenmethylamine, low level of benzaldehyde and presence of almost only benzylalcohol, that is the product of the benzaldehyde reduction by the constitutive alcohol dehydrogenases of the *E.coli* host cells.

To partially overcome the problem of the low stability of the substrate in the water phase an alternative approach has been tried.

The use of a second organic phase is a widely applied technique in biocatalysis to stabilize substrates and products, as well as tool to enhance the overall yield of a biocatalyzed reaction (Morgan, 2004). Bioreductions have been started topping the water phase, containing cells and glucose to reload the cofactors, with a second organic phase where the benzylidenmethylamine substrate was solved. As second phase both hexane and ethyl acetate have been tried, to check the possible role played by the kind of solvent.

Even in this case no bioreduction has been observed, by any mean of reaction conditions. However the analysis of the time course of the reaction revealed that the substrate benzylidenmethylamine was more stable in the biphasic system. The presence of

benzaldehyde was lower than in the reaction in only water phase and the production of benzylalcohol was prevented to a great extent by the presence of the organic solvent.

Conclusions for bioreduction with enoate reductases

An optimized *E.coli* strain, that reduces cinnamic acid, was tested as possible candidate for the promiscuous bioreduction of imines.

For every attempt a control reaction was set, that means that for every condition applied for reduction of the imine leading compound, a parallel reaction was performed under the same conditions for the reduction of cinnamic acid.

In every control reaction the cinnamic acid conversion was complete in 2-5 hours using the optimal conditions for cinnamic acid reduction, otherwise in maximum 18 hours with different than optimal reaction conditions.

Instead secondary amine was never detected by any mean of reaction conditions.

Studies of the stability in water of the applied starting material (benzylidenmethylamine) were made, demonstrating degradation.

In parallel also the stability of the desired product was investigated to assure that no degradation of the possibly formed product (secondary amine) could have smothered the investigated promiscuous bioreduction. No hydrolysis of the commercially available secondary amine was detected in water in the pH range 4-11 even at prolonged incubation times (till two weeks) at room temperature.

One of the possible explanations about the lack of conversion could involve the protonation state of the investigated imine: when it is solved in water, the carbon nitrogen double bond undergoes protonation. The measured pKa of the studied imine was found in literature (Alex, 1991) to be ca. 23 and this data was confirmed by laboratory analysis. A pKa of ca. 23 means that by any mean of pH in the water phase, the imine remains always in the protonation state (figure 3.17) and this could prevent interaction with the tested enoate reductases. This hypothesis is currently under investigation by Prof. Halling, at the University of Glasgow, Scotland.

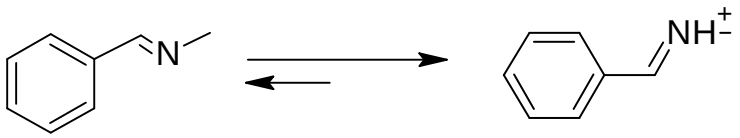


Figure 3.17: The protonation to which undergoes the benzylidenmethylamine when is solved in water.

The conclusion that benzyilidenmethylamine was not susceptible of bioreduction within the performed reaction conditions was anyway drawn.

Bioreduction attempts of benzaldoxime with recombinant enoate reductases

Since benzaldoxime shows steric and electronic similarities with both cinnamic acid and benzylidenmethylamine (figure 3.18), benzaldoxime was identified as possible promiscuous substrate for enoate reductase and then it was reputed worth to be tested with the developed and optimized cinnamic acid reductase system.

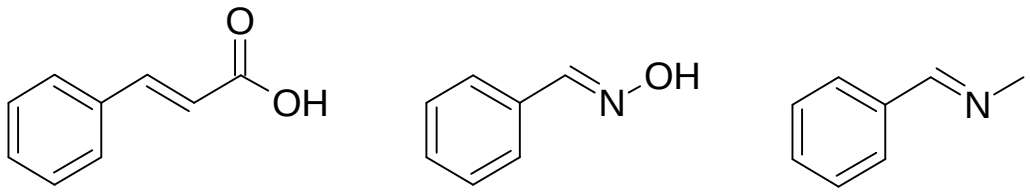


Figure 3.18: compounds used as substrates in the test with the recombinant enoate reductases: on the right side cinnamic acid, the natural substrate for the enoate reductase; in the middle benzaldoxime, a molecule identified as possible promiscuous substrate for the carbon nitrogen double bond reduction, on the left side benzylidenmethylamine, the leading compound applied in the screening to identify possible promiscuous imino bioreduction.

Benzaldoxime was reputed worth to be tested for three main reason. As first It bears a carbon nitrogen double bond, the target of the present studies on promiscuity. Secondarily, the hidroxyl group renders the carbon in alpha position to the nitrogen more electrophilic than the correspondent carbon in the previous tested benzylidenmethylamine and hence a different reactivity is expected. Moreover, the benzaldoxime is known from literature to be stable in water, without undergoing any hydrolysis.

The bioreduction of benzaldoxime was performed using the same reaction's setup applied for the reduction of benzylidenmethylamine (biomass of E.coli harbouring the enoate reductase and the glucose dehydrogenase for the cofactor recycle, different pH and temperature, buffer and biphasic system as solvent), as resumed in table 3.5.

Table 3.5: Resume of the different reactions conditions tried in the bioreduction attempts of benzaldoxime with the cloned enoate reductases. CaERI: *Clostridium acetobutillicum* enoate reductase I; MER: enoate reductase isolated from the metagenome.

Enzyme	**pH**	**Temperature**	**Biphasic system**	**conversion**
CaERI	6; 7; 8	30; 37; 45; 55 °C	No	No
CaERI	6; 7; 8	30; 37; 45; 55 °C	Yes	No
MER	6; 7; 8	30; 37; 45; 55 °C	No	No
MER	6; 7; 8	30; 37; 45; 55 °C	Yes	No

The reaction was monitored via HPLC, using commercially available compounds as standards.

No product formation was detected by any means of reaction's conditions.

Conclusions for the bioreduction of benzaldoxime with enoate reductases

The recombinant overexpressed enoate reductase didn't show to be active toward the desired substrate benzaldoxime. The experiments have been performed using the same procedure already applied in the bioreduction of benzylidenmethylamine. Even in the case of reduction of benzaldoxime, for every applied reaction condition a control reaction was performed, using cinnamic acid as substrate at the same concentration tried with benzaldoxime. As mentioned with the reaction's controls for benzylidenmethylamine reaction, the conversion of cinnamic acid was complete in few hours, except only some cases that reached full conversion overnight. Due to the stability of benzaldoxime in the water phase, the reaction time was prolonged till 3 days, compared to the 24 hours in the study with benzylidenmethylamine.

3.8: Application of recombinant CPCR on promiscuous reduction of carbon nitrogen double bond

Bioreduction attempts of benzylidenmethylamine with recombinant CPCR

Another class of enzymes that was selected as possible target for the promiscus carbon nitrogen double bond reduction was the carbonyl reductases.

This enzyme class was chosen by an analogy with the chemical catalyst chemistry. Usually, chemical catalysts that reduces carbonyl groups can reduce also iminic bonds under certain reaction conditions (Tang, 2003). Prompted by this analogy, the hypothesis of testing carbonyl reductases as possible target for the promiscuous imine reduction was made.

As leading compound was taken once more the benzylidenmethylamine and the natural substrate was chosen to be acetophenone. The three dimensional structures of both the molecule are shown in figure 3.19.

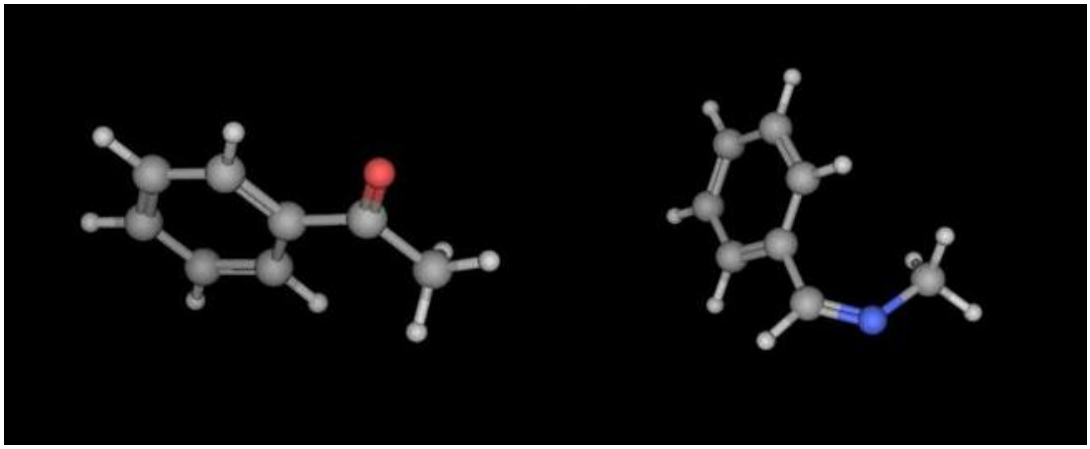


Figure 3.19: Three dimensional representation of the natural (acetophenone, left side) and promiscuous (Benzylidenmethylamine, right side) substrate for carbonyl reductases.

In order to rationalize the approach an *in silico* screen was made, in collaboration with Dr. Braiuca at the Trieste University, Italy.

Dr. Braiuca docked the promiscuous substrate benzylidenmethylamine in the active site of some carbonyl reductases and as feedback resulted that the active site of the cloned *Candida parapsilopsis* carbonyl reductase (CPCR) could harbor either the iminic substrate, as well as its reduced product, the secondary amine, as shown in figure 3.20.

Prompted by this observation reduction experiments have been performed with the cloned CPCR in the attempts in reducing imines.

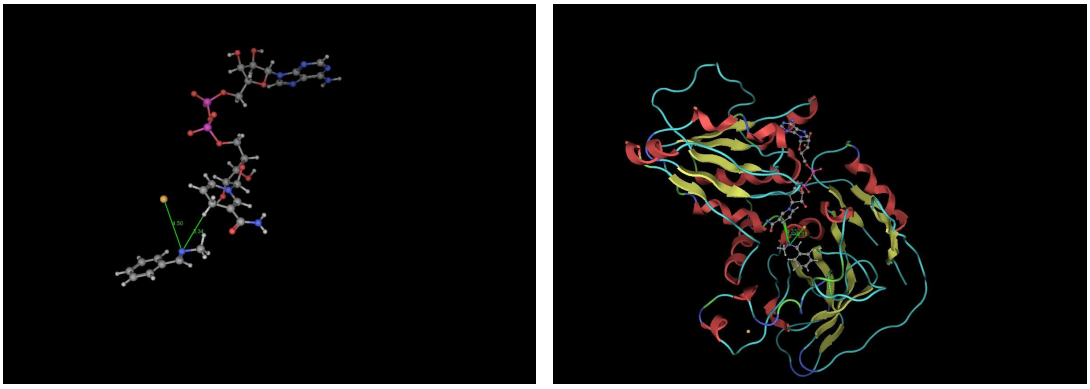


Figure 3.20: On the left side there is the promiscuous substrate benzylidenmethilamine docked in the active site model of the CPCR. On the right side there is the desired reduction's product, the secondary amine, docked as well in the model of the CPCR.

The bioreduction of the carbon nitrogen double bond of the studied substrate was tried under various conditions: in buffer, in biphasic system and also in pure organic solvent.

Reduction attempts in buffer

The first attempts were made in water phase, in a cuvette directly inside an UV spectrophotometer: in literature, the bioreductions of the carbonyl group are usually monitored by UV detection at 340 nm, in order to follow the consumption of NADH cofactor during the reaction.

In this specific case, a similar UV assay, already described for acetophenone (ref), has been applied.

Intriguingly, when the first reactions were tried, a decrease of absorbance was observed; since the decrease was proportional to the enzyme quantity added, it was believed that a reaction was truly happening. Nevertheless, when the reaction was followed and analyzed via GC, other peaks than the secondary amine were found.

With the help of GC-MS it has been discovered that the promiscuous substrate benzylidenmethylamine was undergoing hydrolysis in water. One of the hydrolysis products, namely benzaldehyde, was one of the best substrates for the carbonyl reductase, and was converted by the CPCR to benzylalcohol. This reaction happened with

the consumption of NADH, thus leading to a decrease in the NADH concentration detected with the UV spectrometer. The reaction scheme in figure 3.21 shows this side reaction.

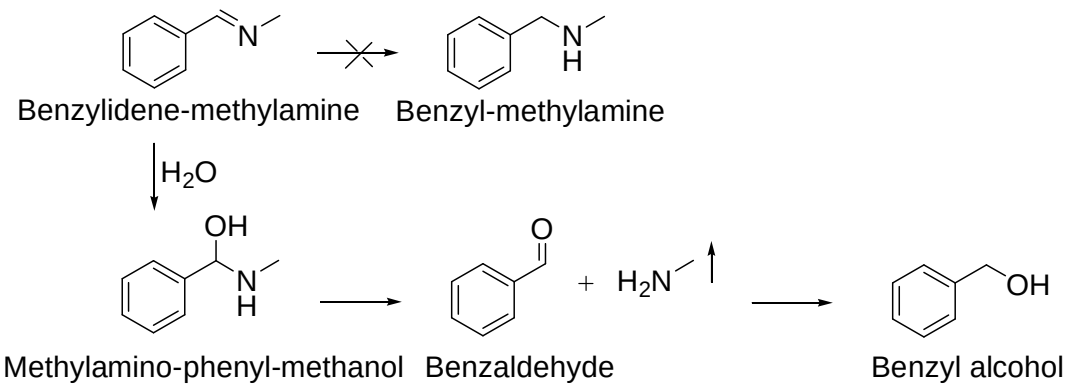


Figure 3.21: the hydrolysis pathway of benzylidenmethylamine in water phase. In the case of bioreduction attempts with recombinant CPCR it was observed a decrease of the NADH absorbance within the reaction, an indication that the enzyme was using the cofactor to reduce double bonds. A deeper analysis of the reaction via GC-MS showed that the benzaldehyde (hydrolysis product of benzilidenmethylamine) was fastly reduced by the CPCR to benzylalcohol, with the consumption of NADH.

Reduction attempts in biphasic system

In order to overcome the instability of the substrate in water phase, the reaction has been tried in a two phase system. The use of a second organic phase is a widely applied technique in biocatalysis to stabilize substrates and products, as well as tool to enhance the overall yield of a biocatalyzed reaction (Morgan, 2004).

As described in the section 2.8.3, bioreductions of benzylidenmethylamine have been started topping the water phase, containing the cloned CPCR and NADH as cofactor, with a second organic phase where the benzylidenmethylamine substrate was solved. As second phase both hexane and ethyl acetate have been tried, to check the possible role played by solvents with different properties.

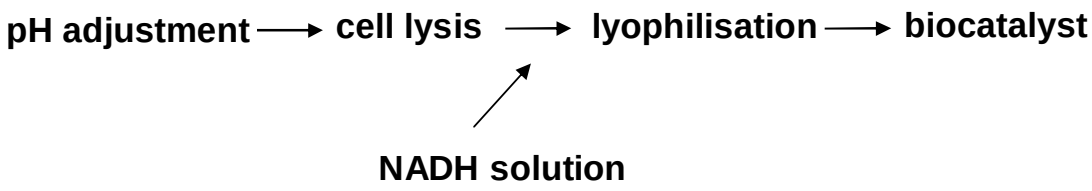
The reaction was followed by GC, using authentic standards for all the reaction products.

Compared with the reaction in water phase, the effect of a second organic phase maintained the promiscuous substrate benzylidenmethylamine stable for a longer time, and also the overall benzylalcohol formation was reduced. Nevertheless, no amine formation was detected within the reaction time.

At this point it has been postulated that maybe the water content of our reaction systems played a crucial role, either by hydrolyzing the promiscuous substrate, or by changing the protonation level of the substrate, as mentioned above in this paragraph.
In order to understand if the water presence in our reaction system was preventing the promiscuous enzymatic reductions, a series of experiment has been performed where the water content was minimized.

Reduction attempts in pure organic solvent

In order to understand if water presence in the reaction system hampered the promiscuous carbon nitrogen double bond reduction, experiments with the lyophylized powder of a CPCR preparation were performed.
A huge quantity of E.coli biomass (1 g wet biomass) containing the expressed and active CPCR was resuspended in buffers at different pHs, were lysed, NADH was added and frozen, subsequently lyophilized and used as direct biocatalyst for the studied reaction, as explained in scheme 3.4.



Scheme 3.4: Flow sheet of the procedure used to obtain the biocatalyst to use in organic solvent.

For every reaction condition tried a control was run in parallel, consisting in the same reaction system but containing the natural substrate acetophenon. The reaction setups in details are given in the section 2.8.2.
Reaction started adding the investigated substrate and isopropanol to recycle the cofactor dissolved in organic solvent. Hexane and ethyl acetate were chosen to discover if the kind of solvent could play a role in the bioconversion. The reactions were followed by GC analysis at different reaction intervals.
In all the experiments no secondary amine formation has been detected. The controls reaction showed activity for the reduction of acetophenon to S-phenylethanol at some pHs, but not in all the studied interval. Table 3.6 clarifies the results.

Table 3.6: pH memory for the biocatalyst preparation used in the promiscuous reduction of benzylidenmethylamine and the control reduction of acetophenone. The preparation used in the reaction setup showed to be reactive for the control reaction (acetophenone reduction) at some pHs.

pH studied	5.5	6.5	7.5	8.5	9.5	10.5	11.5	12.5	
Benzylidenmethylamine	No	No	No	No	No	No	No	No	
Acetophenone	No	Yes	Yes	Yes	Yes	No	No	No	

The experiments showed that the enzyme CPCR was active in some of the pH studied for the reduction of acetophenon to phenylethanol in pure organic solvent within the reaction's setup used, nevertheless the formation of secondary amine in the studied promiscuous imine reduction has not been observed.

Conclusions for bioreduction attempts of benzylidenmethylamine with recombinant CPCR

During the present studies, the reduction of imines has been investigated as possible promiscuous activity of *Candida parapsilopsis* carbonyl reductase (CPCR); in particular, the conversion of benzylidenmethylamine to the corresponding secondary amine has been selected as target reaction.

By GC-MS analysis, it has been demonstrated that the selected substrate benzylidenmethylamine was hydrolyzed to benzylaldehyde, performing the reaction in water phase; subsequently, benzylaldehyde was reduced to benzylalcohol by the CPCR, thus driving the reaction toward the hydrolysis.

Performing the reaction in biphasic system, the hydrolysis of the substrate needed longer time to occur, but anyway no product formation was observed.

In order to avoid the hydrolysis of the substrate and to verify a possible "pH memory" effect on CPRC, different batches of biocatalyst were prepared in different pHs and tested in pure organic solvent, in water free system.

In this case neither products of hydrolysis were observed, nor the desired secondary amine.

In parallel, the same reactions were performed on the natural substrate acetophenone, verifying reduction activity under the tested experimental conditions.

The experimental results didn't match the prediction obtained by docking the investigate substrate benzylidenmethylamine and the corresponding reduction product in the active side of CPRC.

In order to explain the discrepancy between the prediction of the *in silico* studies and the observed experimental results, different hypotheses have been formulated.
Since it is known by literature (Dr. Bhattacharjee, PhD thesis, 2006) that the CPCR is able to perform both the reduction of acetophenone to phenylethanol as well the opposite oxidation, it has been thought that, in the case of the selected imine, the reaction could work only from the oxidation site, thus the oxidation of benzylmethylamine to benzylidenmethylamine has been investigated.
Since product formation (benzylidenmethylamine) has been detected in none of these experiments, a further hypothesis has been formulated: maybe the active site of the CPCR reduced the imine to the corresponding secondary amine, but this later was not released in the bulk phase due to high affinity and interaction with the active site. In order to verify this hypothesis, acetophenone reduction in water phase has been performed in presence of the secondary amine and monitored by spectrophotometer.
By classical kinetic studies, it has been observed that the secondary amine added in the reaction media played indeed the role of a not-specific inhibitor.
After this result, the question about the reduction of imines as possible promiscuous activity of *Candida parapsilopsis* carbonyl reductase remained open.

<u>Reduction attempts of benzaldoxime with recombinant CPCR</u>
Since benzaldoxime shows steric and electronic similarities with both acetophenone and benzylidenmethylamine (figure 3.22), benzaldoxime was identified as possible promiscuous substrate for the CPCR and then the bioreduction of carbon nitrogen double bond was reputed worth to be tested.
Benzaldoxime was reputed worth to be tested for three main reason. As first It bears a carbon nitrogen double bond, the target of the present studies on promiscuity. Secondarily, the hydroxyl group renders the carbon in alpha position to the nitrogen more electrophilic than the correspondent carbon in the previous tested benzylidenmethylamine and hence a different reactivity is expected. Moreover, the benzaldoxime is known from literature to be stable in water, without undergoing any hydrolysis.

The bioreduction of benzaldoxime was performed only in water phase at different pHs. In any attempt, product formation has not been detected by any means of reaction's conditions, even prolonging the reaction time to three days and adding fresh cofactor to the reaction after two days.

The reaction was monitored via HPLC, using commercially available compounds as standards.

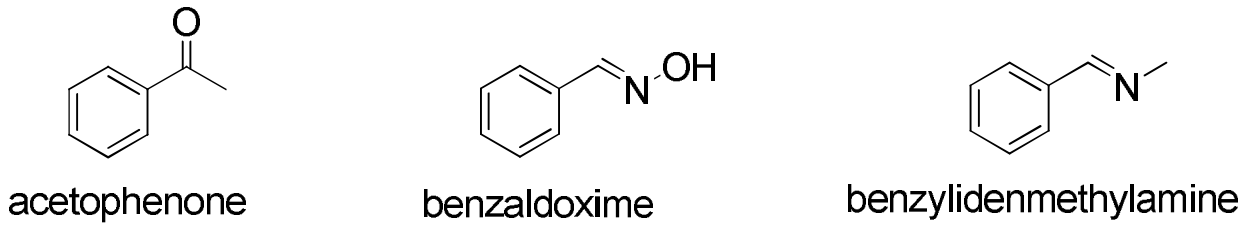


Figure 3.22: compounds used as substrates in the test with recombinant CPCR: acetophenone, the natural substrate for the CPCR; benzaldoxime, a molecule identified as possible promiscuous substrate; benzylidenmethylamine, the compound docked *in silico* in the active site of the CPCR and investigated as promiscuous substrate for the promiscuous reduction of the double bond carbon nitrogen.

Conclusions for bioreduction attempts of benzaldoxime with recombinant CPCR

The present study is focused on the investigation of the reduction of carbon nitrogen double bond as promiscuous activity for *Candida parapsilopsis* carbonyl reductase (CPCR).

Since the reduction of the imine system was not successful, the reaction was performed on an oxime system; in particular, the conversion of benzaldoxime to the corresponding hydroxyphenylmethanamine has been selected as target reaction.

Under the reaction's conditions tested no reduction of the carbon nitrogen of the oxime has been detected.

3.9: Low throughput screening with microbial cells collections for the reduction of carbon nitrogen double bond of benzylidenmethylamine and benzaldoxime

In order to isolate a new biocatalyst able to perform the reduction of imines (figure 3.23), wild type cells of several microorganisms and yeasts have been screened in low throughput screening.

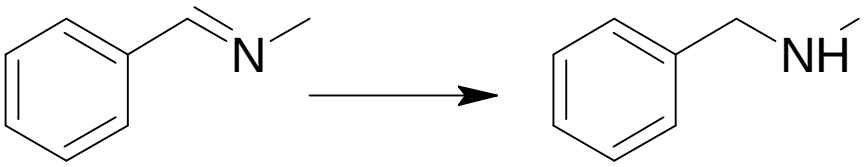


Figure 3.23: The investigated reaction: bioreduction of benzylidenmethylamine to benzylmethylamine.

Resting cells of the studied microorganisms were produced as described in the session 2.2. The cells were resuspended in sodium phosphate buffer pH 7.0 0.1M and transferred in GC glass vials. . The reaction started topping the cells suspension with an equal amount of n-hexane in which the substrate imine was dissolved in concentration of 0.01M, as described in the section 2.5.1. The reactions were monitored via GC using commercially available compounds as standars, but no product has been detected.
The list of the investigated microorganism is in table 3.6.

Table 3.6: List of the microbial strains tested for the reduction of benzaldoxime. No conversion has been detected with any of the strains.

Sporomusa termitida	DSM 4440
Acetobacterium woodii	DSM 1030
Lactobacillus brevis	25a BAGKF
Lactobacillus bulgaricus	DSM 20081
Lactobacillus casei	NCDO 15[1]
Lactobacillus casei ssp. casei	DSM 20011 T
Lactobacillus delbrückii	DSM 20074
Lactobacillus kefir	DSM 20587
Lactobacillus plantarius	DSM 20174
Lactococcus lactis ssp. cremoris	DSM 20069 T
Clostridium acetobutylicum	DSM 1731
Clostridium buryricum	DSM 10702
Clostridium celerecrescens	DSM 5628
Clostridium pasterianum	DSM 525
Candida boidinii	DSM 70024
Candida parapsilosis Jul.00, Jan.02	DSM 70125
Candida cylindracea, syn. rugosa	DSM 2031

Candida rugosa	DSM 2031
Candida tropicalis	DSM 5991
Debaryomyces hansenii	DSM 70590
Hansenula anomala	CBS 172 blau !!!
Hansenula capsulata	CBS 1993
Hansenula polymorpha	Wildstamm BioVT
Brauereihefe HeBru	
Brauereihefe K 35	
Kluyveromyces marxianus	St 5, Lac´b
Lipomyces kononenkoae	DSM 70302
Pichia haplophilia	DSM 70365
Saccharomyces cerevisiae	
Saccharomyces cerevisiae	DCL-St., DC´
Saccharomyces cerevisiae	Invertasehefe
Saccharomyces cerevisiae	MCC 109 RHO0
Saccharomyces cerevisiae	DSMZ 1333 Wildtyp
Saccharomyces cerevisiae	DSMZ 1333 rho^0-Mutante
Saccharomyces cerevisiae	CBS 1172 Wildtyp

In order to isolate a new biocatalyst able to perform the reduction of benzaldoxime (figure 3.24), wild type cells of several microorganisms and yeasts have been screened in low throughput screening.

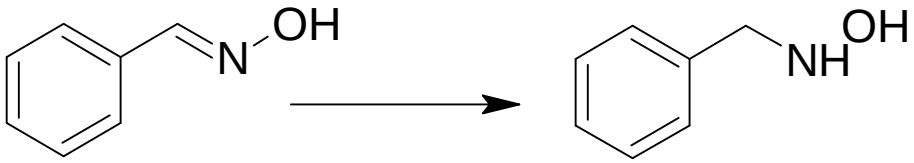


Figure 3.24: The investigated reaction: bioreduction of benzaldoxime to hydroxyphenylmethanamine.

Resting cells of the studied microorganisms were produced as described in the session 2.2. The cells were resuspended in sodium phosphate buffer pH 7.0 0.1M containing benzaldoxime 0.01M. A solution of sugars was added to recycle cofactors and the reactions were incubated at 30 °C for few days. The reactions were monitored was

followed via HPLC using commercially available compounds as standars, but no product has been detected.

The list of the investigated microorganism is in table 3.7.

Among the tested strains, also *Saccaromices cerevisie* was applied in the investigated reaction, because its capability in the reduction of benzaldoxime has been already reported in literature (Chimni, 1998). Nevertheless, in the tested reaction conditions, this result was not reproduced, also after applying the same experimental procedure described in literature.

Conclusions on reduction of carbon nitrogen double bond in oximes

In order to isolate a new biocatalyst able to perform the reduction of the carbon nitrogen double bond of benzaldoxime, wild type cells of several microorganisms and yeasts have been screened in low throughput screening.

All the reactions have been monitored for days via HPLC, but in any case the reduced product hydroxyphenylmethanamine has not been observed.

The reactions were performed under different conditions. Moreover, the cells of every applied strain were grown on their specific broth supplied with 1 mM benzaldoxime as possible inducer of the activity; in order to enhance the detection limits by concentrating the organic compounds present in the cell broths, the cultivation broth was extracted twice with an equal volume of ethyl acetate, dried under nitrogen flow and the solid obtained resuspended in H_2O:CH_3CN mixture and analyzed via HPLC.

By any strain the desired product was not observed.

The bioreduction of the carbon nitrogen double bond in benzaldoxime has been unsuccessful tested also with the recombinant enoate reductase.

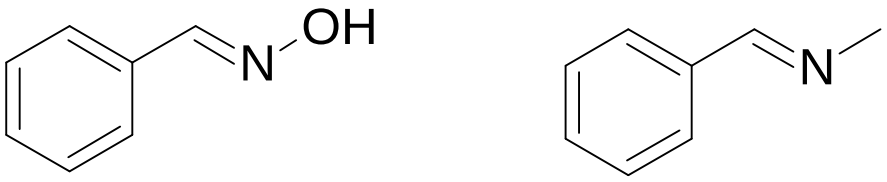


Figure 3.25: Benzaldoxime on the left side, benzylidenmethylamine on the right side. Both the substrates were applied in test reactions for investigating the promiscuous reduction of the carbon nitrogen double bond, using whole cells, enoate reductases and carbonyl reductases as biocatalysts.

3.10: Isolation of a putative epoxide hydrolases from metagenome

Screening for epoxide hydrolases

During these years of PhD, our laboratory was interested in different projects; one of them was focused on the isolation of new epoxide hydrolases. Within this frame the screening of the metagenomic library prepared during the present study was carried out, leading to the isolation of a new putative epoxide hydrolase.

The obtained library of metagenomic DNA cloned in the pZero-2 vector (Invitrogen, USA) was washed away from the transformation plates after the colonies were transferred in deep multiwell plates with the Genetix colony picker (company, town, country), resulting in a pool of clones for every transformation plate. The pool of clones from every transformation plate was washed twice with sterile phosphate buffer (NaH2PO4/Na2HPO4, pH 7.0, 50 mM) and used to inoculate assay tubes containing 5 mL of selective media for epoxide hydrolases (LB broth supplemented with 0,05% glycidol vol/vol and 50 µg/mL kanamycin)(for details see material and methods).

In this case the employed screening technique was not laborious, because the used medium was selective itself, since it contained glycidol as selecting agent, known for its inhibition of cell growth (Reetz, 2006). Thus, a clone expressing an enzyme able to hydrolyze glycidol to its corresponding diol (glycerol, figure 3.26), will grow in this medium and could be detected by turbidity formation directly in the assay tubes. On the contrary, clones lacking or expressing incorrectly an epoxide hydrolase will not hydrolyze the glycidol, consequently no growth will be observed.

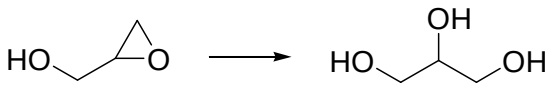


Figure 3.26 Glycidol (left side) inhibits cell's growth; If glycidol is hydrolyzed to glycerol due to the action of a epoxide hydrolase the reaction product simply enters the metabolic cycles of the cell as carbon source.

Among all the assay tubes that were inoculated with the metagenomic clones only three (named clones "A", "B", "C" in figure 3.27, left side) showed turbidity after 48 hours incubation at 30 °C on an orbital shaker.

The cells were pelleted and the plasmids recovered with a commercial kit (Plasmid mini prep, Eppendorf, Germany). Consequently, restriction analysis was employed using two restriction enzymes flanking the multiple cloning site of the vector (figure 3.27, left side), thus releasing the cloned insert DNA. The obtained plasmids from the three positive clones were retransformed in *E.coli* TOP10 cells and used to inoculate assay tubes with the same selective medium in order to reconfirm the activity towards glycidol hydrolysis.
After the second transformation only the clone named B confirmed its activity toward glycidol, the other clones A and C instead didn't grow.
The restriction analysis of the second transformant of the clone B showed the same pattern as before retransformation (figure 3.27, right side).

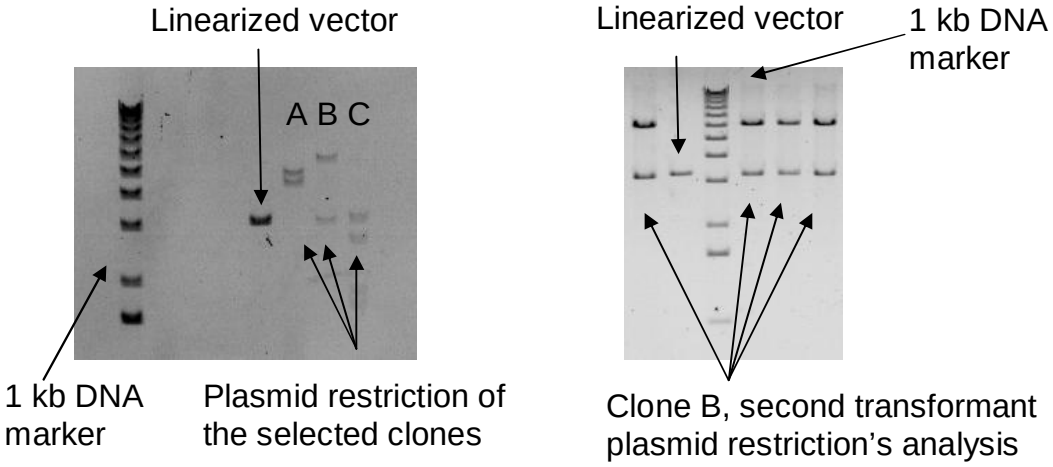


Figure 3.27: Restriction analysis of the clones able to grow on the selective medium for epoxide hydrolase. On the left side the restriction analysis of the clones A, B, C. On the right side the plasmid analysis of the second transformants of the clone B, the only one that confirmed its activity.

Bioinformatic Analysis of the cloned metagenomic DNA

The plasmid of the clone B revealed a insert size of circa 6.5 kb and was fully sequenced at MWG Biotech (dito, see above,Germany).
The completely sequenced 6.5kb insert was analyzed for open reading frames (ORFs) using the Vector-NTI package (Invitrogen,USA) and annotated by simililarity using the BLAST-tool available at www.expasy.org. The results of the analysis with respect to all open reading frames in the sequenced DNA strand is shown in figure 3.28.

All Open Reading Frames

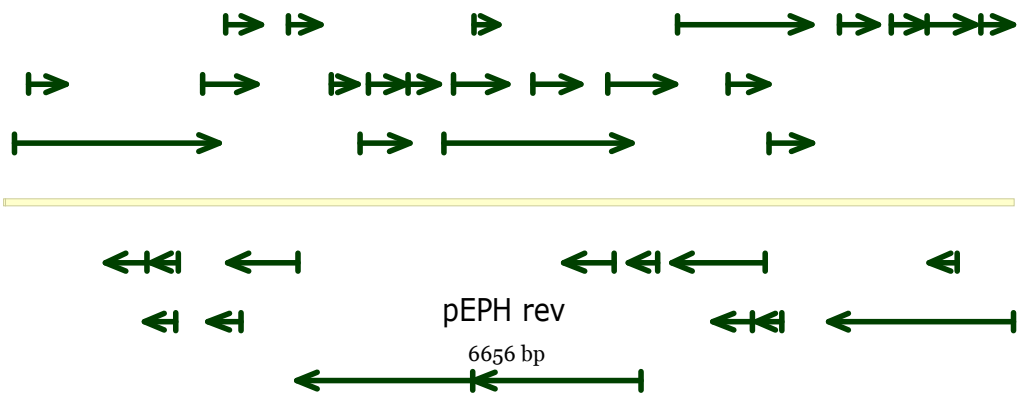


Figure 3.28: Graphical representation of all the open reading frames found in the sequence analysis of the circa 6.5 kb DNA fragment isolated from the metagenome.

Using the BLAST-tool an ORF could be identified which showed similiarity to “metal dependent hydrolases”, for a whole representation of the ORFs that showed similarity with already published genes see figure 3.30. The complete annotation of the DNA region containing this putative hydrolase is shown in figure 3.29. The putative hydrolase possesses the highest degree of similarity with a metal dependent hydrolase (YP_004888) of *Thermus thermophilus* HB27 (20% identity, 31% similarity). Nevertheless, those percentages were anyway too low to clearly assign the epoxide ring hydrolysis to the putative metal dependent hydrolase gene found within the 6.5 kb DNA fragment.

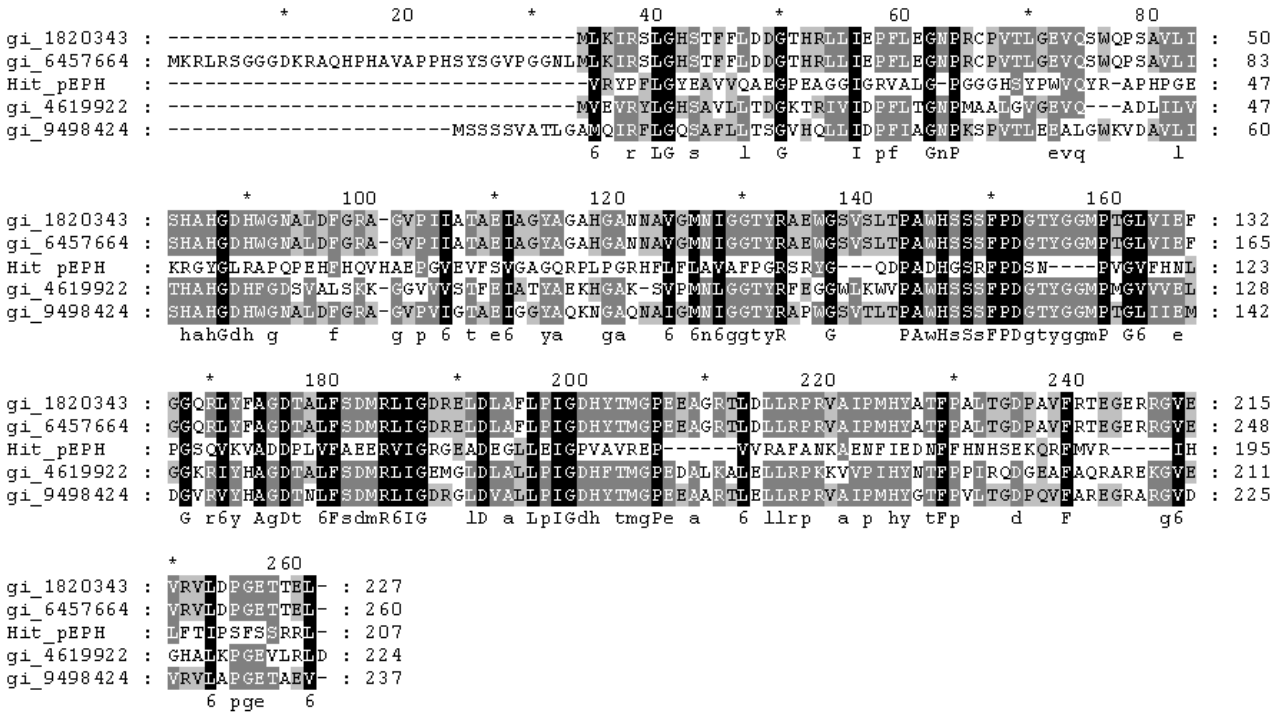


Figure 3.29: Alignment of Metal Dependent Hydrolases(BLAST) with pEPH Hit

Significant BLAST HITS

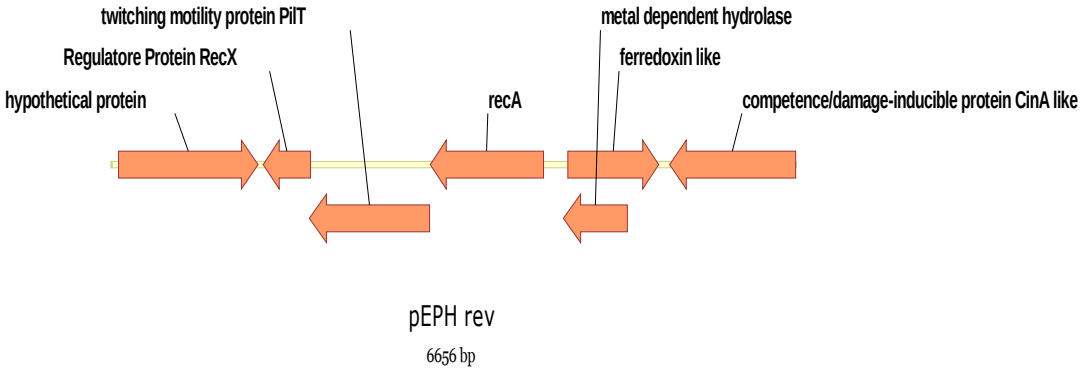


Figure 3.30: The arrows represent ORFs with significant hits found in the BLAST analysis for the sequence of the circa 6.5 kb DNA fragment isolated from the metagenome.

Insertional gene inactivation by transposon integration

As the BLAST analysis of the sequence of the DNA fragment didn't lead to any clear indication about which gene was finally responsible for the observed activity, a DNA

transposon was inserted randomly within the isolated 6.5 kb of DNA fragment to knock out the specific gene related to the ability of the clone to grow on the toxic substrate glycidol.
This technique has been already reported to be successful in silencing the desired activity, allowing the isolation and identification of the gene of interest (HyperMu™ MuA Transposase, Epicentre, USA).
The new library of transformants, obtained by the random insertion of the transposon in the 6,5 kb DNA fragment, was manually picked and used to inoculate in parallel two different deep-well plates at the same position.
One multiwell plate contained the epoxide hydrolase selective medium, the other contained normal LB as control. After an overnight incubation at 37 °C in a shaker for microtiter plates, a plasmid was isolated from a clone that was not able to grow on the selective medium, thus having the transposon inserted in the gene responsible for the previously observed activity. The plasmid was isolated using a commercially available kit (Eppendorf, Germany), by, simply recovering the *E.coli* cells from the control multiwell plate, (that contained LB broth), at the same well's position where in the assay plate (with the selective medium) the clone was not able to grow, as shown in figure 3.31.

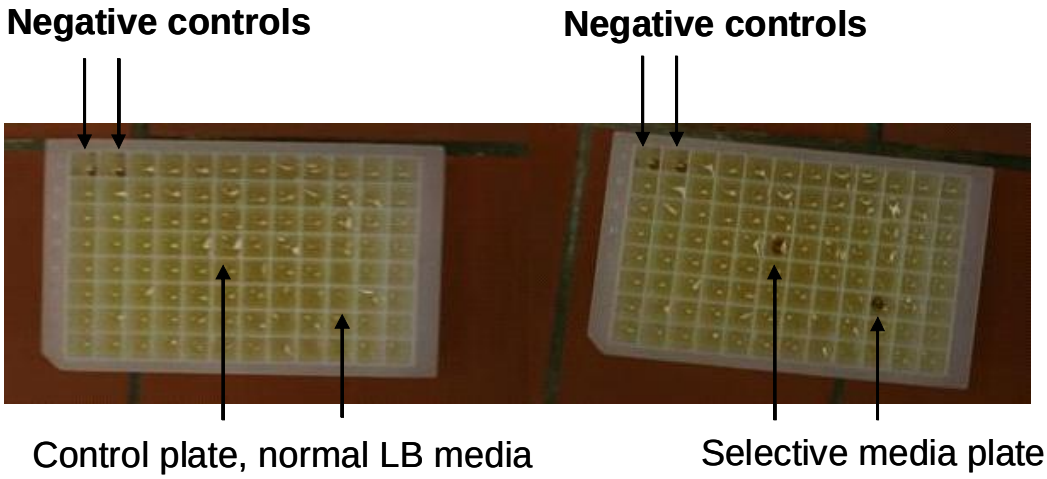


Figure 3.31: On the left side the control deep well plates where the obtained mini-library with the random transposon insertion was cultivated. On the right side the assay deep well plate with the selective media for epoxide hydrolysis. The clones that didn't grow in the pointed positions in the selective media plate was recovered from the same position in the control plate.

The gene responsible for the ability of the clone to grow on the toxic epoxide compound glycidol was singled out by a sequencing-run up- and downstream from the transposon insertion by using specific primers designed for the transposon, as described by the manufacturer (HyperMu™ MuA Transposase, Epicentre, USA.) Figure 3.32 shows the insertion point for transposon in the original 6.5 kb DNA fragment.

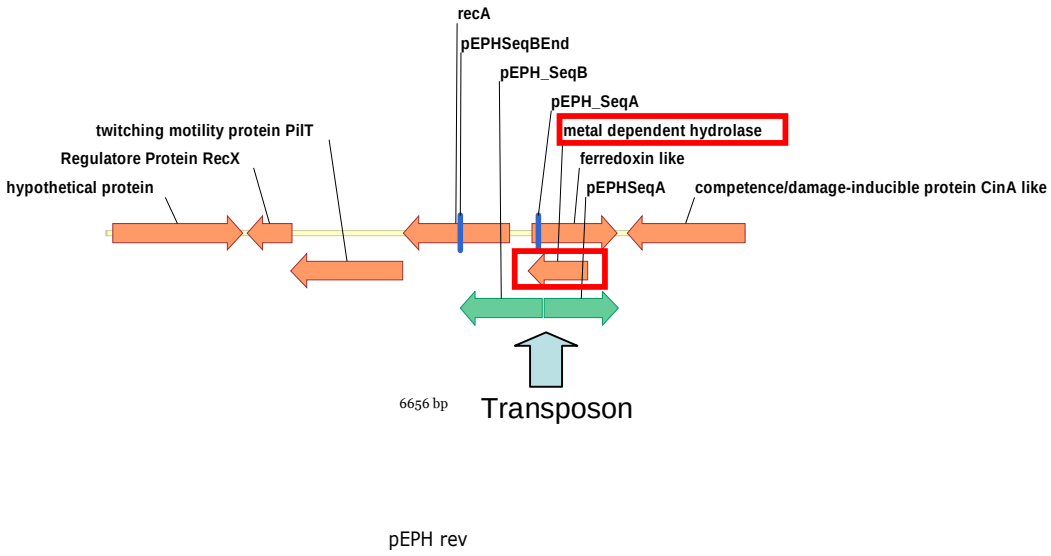


Figure 3.32: The position in the 6.5 kb DNA fragment where the transposon was inserted and subsequently the activity was knocked out.

Cloning and expression of the putative metal dependent hydrolase

In order to clone the putative epoxide hydrolase different sets of PCR primers were designed (see materials and methods) and employed to PCR amplify the desired gene.

Two DNA fragments of the desired size were obtained and cloned into the pET22b+ vector. One PCR product (containing a stop-codon at the 3' end of the gene) was cloned in pET22b+, thus resulting in the transcription/ translation of the native gene sequence from the pET22b+ cloning vector without the addition of any tag. The obtained plasmid was named pEH. The second PCR product, lacking a stop codon at the 3' end of the gene was similarly cloned into pET22b+ thus resulting in the addition of a Hexa-Histidine-Tag to the C-Terminus of the protein. Correspondingly, the obtained plasmid was named pEH6His.

The correct cloning of the genes was confirmed by DNA sequencing at MWG Biotech.

After protein expression, SDS-PAGE analysis of whole cell extracts revealed that the protein could be expressed in significant amounts (figure 3.33).

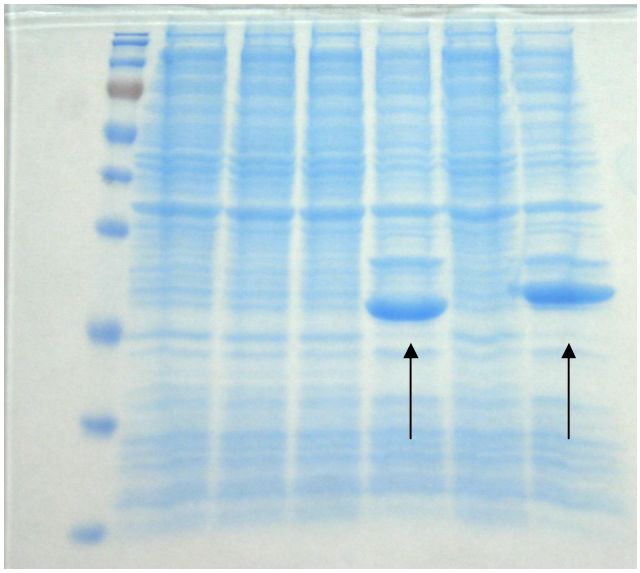


Figure 3.33: SDS-PAGE anaylsis of the putative epoxide hydrolase expressed in *E.coli*. The arrow on the left indicates the expression of the native protein (without His-Tag), The arrow on the right indicates the lane with expression of the respective Hexa-His-Tag fusion protein. *E.coli* JM109 (DE3) cells were induced with 0.5 mM IPTG at 30°C for four hours.

The properties of the protein, derived by bioinformatics tools, are:

Sequence: 209 Aa, Theoretical pI/Mw: 6.60 / 23095.99

The aminoacidic sequence of the cloned protein is:

MRYPFLGYEAVVQAEGPEAGGIGRVALGPGGGHSYPWVQYRAPHPGEKRGYGLRAPQ

PEHFHQVHAEPGVEVFSVGAGQRPLPGRHFLFLAVAFPGRSRYGQDPADHGSRFPDSN

PVGVFHNLPGSQVKVADDPLVFAEERVIGRGEADEGLLEIGPVAVREPVVRAFANKAENF

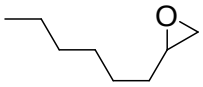
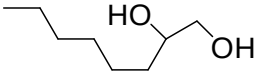
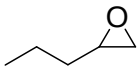
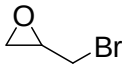
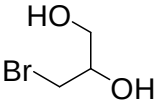
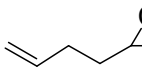
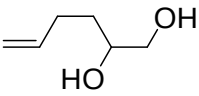
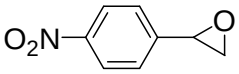
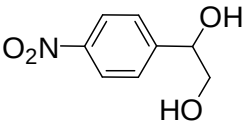
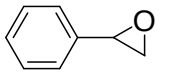
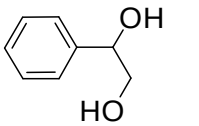
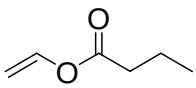
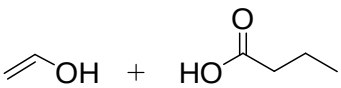
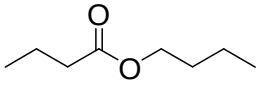
IEDNFFHNHSEKQRFMVRIHLFTIPSFSSRRLLE

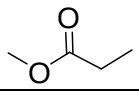
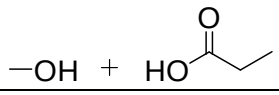
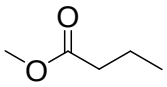
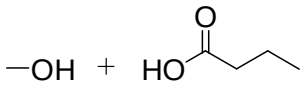
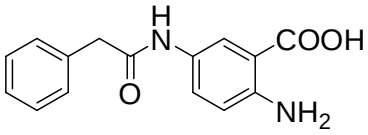
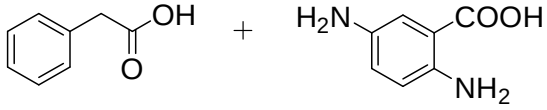
Hydrolysis attempts with the overexpressed putative epoxide hydrolase

E.coli JM109 (DE3) was transformed with the plamids pEH and pEH6His. Biomass of both the clones expressing the proteins was produced and tested for activity.

The table 3.7 summarizes all the substrates that were tested in the respective hydrolysis reactions. All reactions were performed in sodium phosphate buffer (NaH2PO4/Na2HPO4, pH 7,0, 0.05M) at 30 °C and 37 °C with the whole cell extracts as biocatalyst. The expressed protein showed neither activity on any of the epoxide substrates nor on any of the esters that have been tested for hydrolysis.

Table 3.7: Substrates tested with the putative epoxide hydrolase

Tested substrate	Expected product(s)
	
	
	
	
	
	
	
	

In order to understand to which class of enzymes the newly isolated protein belongs, several additional activity tests were performed, all without positive result.

In more detail, the cells overexpressing the putative epoxide hydrolase have been tested in tributyrin, skimmed milk or egg-yolk agar-plate tests in order to detect esterolytic/lipolytic or proteolytic or phospholipolytic activity. Tests on the hydrolysis of p-nitro-styrene oxide, by using either a filter paper assay (Zhang, 1986) or UV/VIS spectrophotometric assay (Alkema, 1999), also revealed no activity. Hydrolysis of NIPAB (2-nitro-5-[(phenylacetyl)amino]-benzoic acid), a routinary test to detect amidase activity, was performed without positive result.

The lack of any detectable hydrolytic activity in the cells overexpressing the putative epoxide hydrolase from the metagenome, suggests that more than one gene from the 6.5 kb DNA fragment might be needed to confer the *E.coli* host cells the ability to grow on glycidol.

CHAPTER 4: CONCLUSIONS

Conclusions

The bioreduction of imines is a reaction that nature didn´t evolve. This conclusion is drawn by the fact that imines are not stable in water (ref.).
However, biocatalysis can overtake this drawback with the enzyme promiscuity concept (as described in section 1.4).
Within this research project the bioreduction of imines has been investigated as possible promiscuous activity of two different reductases families, the enoate reductase family (EC 1.3.1.31) and the carbonyl reductase family (EC: 1.1.1.1).
The decision of targeting these enzyme classes has been based on the existing literature and on substrate analogy for the enoate reductases (see section 3.7) and based on *in silico* docking for the carbonyl reductase (see section 3.8).
As a carbonyl reductase was already available in our research group, an activity based screening for the isolation of enoate reductases was performed within this research project.
As genetic material the DNA of *Acetobacterium woodii* and metagenomic DNA have been used. The DNA of *Acetobacterium woodii* has been cloned into *E.coli* and the obtained library was screened in High Throughput screening format. The same procedure, although with a different vector, has been followed for the metagenomic DNA. As no positive hits were identified within both the screened libraries, the conclusion that the adopted strategy was not successful for the isolation of enoate reductase was drawn.
In order to overcome the problems that arise with an activity based screening (low protein expression and uncorrect folding, as explained in section 3.2 and 3.3), a sequence based screening was performed, designing degenerate primers based on the alignment of the already published enoate reductases. This strategy showed to be successful and led us to the isolation of a new enoate reductase from the metagenome.
The obtained enoate reductase retrieved from the metagenome, together with the cloned enoate reductase from *Clostridium acetobutylicum* and the carbonyl reductase from *Candida parapsilosis*, have been tried in the bioreduction of imines and oximes, under a very diverse reaction conditions.
The formation of the desired product, the secondary amine, has never been detected.

It is our believe that further study in this area can be still performed. A promosing strategy could arise from the combination of *in silico* data from the docking of the imines and oximes in the active sites of both the enzyme families with the rational mutagenesis of the investigated enzymes.

On a practical approach, as it is cumbersome to detect the formated amines with a High Throughput screening, the rational mutagenesis of the active sites of both enzyme families can significantly reduce the amount of clones to screen, thus allowing low throughput screening of the obtained clones.

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Abbreviations

6xHisTag	polyhistidine-tag
ATP	Adenosine-5'-triphosphate
ADH	Alcohol dehydrogenase
APS	Ammonium persulfate
BASF	Badische Anilin- und Soda-Fabrik
BLAST	Basic Local Alignment Search Tool
CaERI	*Clostridium acetobutylicum* Enoate reductase I
CCDB	*E.coli* suicide protein
CERF1	Degenerate primer for enoate reductase
CERR1	Degenerate primer for enoate reductase
CERF2	Degenerate primer for enoate reductase
CERR2	Degenerate primer for enoate reductase
CIAP	calf intestine alkaline phosphatase
CoA	Coenzyme A
CPCR	*Candida parapsilopsis* carbonyl reductase
CTAB	Cetrimonium bromide
DEP	DNA Extraction Buffer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
DNAse	Deoxyribonuclease
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	Dithiothreitol
E. coli	*Escherichia coli*
E.C.	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Admininstration
FID	Flame ionization detector
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
gDNA	Genomic DNA
H^+	Proton

H^-	Hydride ion
HPLC	High Pressure Liquid Chromatography
HTS	High Throughput Screening
IP	Intelectual property
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Unit of length of DNA fragments equal to 1000 nucleotides
LB	Luria Bertani medium
M	Molar
MERI	Metagenomic enoate reductase I
M9	Mineral media
NAD^+	β-nicotinamide adenine dinucleotide, oxydized form
$NADH+H^+$	β-nicotinamide adenine dinucleotide, reduced form
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate, oxydized form
$NADPH+H^+$	β-nicotinamide adenine dinucleotide phosphate, reduced form
NIPAB	6-nitro-3-phenylacetamido-benzoic acid
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PCR	Polymerase chain reaction
pI	Isoelectric point
PWE15	cloning vector
RNAse	Ribonuclease
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
Page	Poliacrylamide gel electrophoresis
SOC	medium
TAE	Tris-Acetate-EDTA
T4	DNA polymerase
Taq	Thermophilus acquaphilae DNA polymerase
TB	Terrific Broth medium
TE	Tris-EDTA buffer
TEA	Trietanolamine
TEMED	N,N,N',N'-Tetramethylethylendiamine

TEN	Tris, EDTA, sodium chloride buffer
TENST	Tris, EDTA, sodium chloride Triton, N-laurylsarcosyl buffer
TLC	Thin layer chromatography
Tris	Tris-(hydroxymethyl)-aminomethane
UV-Vis	Ultra Violet-visible
vol/vol	volume per volume
w/vol	weight per volume

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