# Identification and expression of new microbial oxidoreductases

**Doctoral dissertation**

by

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*„Success doesn't really teach you much, failure teaches you a lot"*

Dr. Bruice Alberts,

co-author of the textbook „Molecular biology of the cell",
Editor-in-chief of *Science* magazine in 2008 – 2013

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## Zusammenfassung

Oxidoreduktasen katalysieren Redoxreaktionen und sind in allen Organismen weit verbreitet. Sie werden wegen ihrer ausgezeichneten Selektivität in vielen Industriezweigen und in der organischen Chemie gerne angewandt. Obwohl eine große Zahl von Oxidoreduktasen bereits beschrieben ist und verwendet wird, ist die Industrie nach wie vor auf der Suche nach Enzymen, die bestimmte Substrate in angestrebte Produkte umsetzen können. Das Ziel dieser Doktorarbeit war es, solche Enzyme zu identifizieren und zu charakterisieren.

Von fünfundzwanzig potenziellen Biokatalysatoren wurden neunzehn Proteine erfolgreich exprimiert: Eine Alkoholdehydrogenase wie auch eine kurzkettige Dehydrogenase-Reduktase aus *Y. lipolytica* CLIB 122 (*Yl*ADH2 and *Yl*SDR), eine Aldehyd-Dehydrogenase aus *Ralstonia eutropha* H16 (*Ras*AlDH) und eine Carboxylat-Reduktase aus *Nocardia iowensis* sp. NRRL 5646 (*Ni*CAR) wurden im Detail untersucht.

Diese Doktorarbeit beinhaltet eine kurze Einführung in der Welt der Oxidoreduktasen sowie einen Überblick ihrer Anwendungsmöglichkeiten. Besonders die industrielle Relevanz der vier obengenannten Enzyme, die im Zuge dieser Doktoratsarbeit untersucht wurde, wird betont.

## Abstract

Oxidoreductases are widely distributed among all branches of the tree of life and catalyze redox reactions. They are extensively used in many branches of industry and organic synthesis, especially, because of their great selectivity. Although many oxidoreductases have been already characterized and are currently applied, the industry is still seeking for enzymes, which can transform particular substrates to the desired products. The aim of this thesis was to identify and characterize such enzymes.

From twenty five proteins, which were chosen as potential biocatalyst, nineteen were successfully expressed and selected aspects were studied in detail: an alcohol dehydrogenase 2 and a short chain dehydrogenase/reductase from *Y. lipolytica* CLIB 122 (*Yl*ADH2 and *Yl*SDR), an aldehyde reductase from *Ralstonia eutropha* H16 (*Ras*AlDH) and a carboxylic acid reductase from *Nocardia iowensis* sp. NRRL 5646 (*Ni*CAR).

This thesis gives a short introduction to the world of oxidoreductases, and provides an overview of their potential application. Especially the industrial relevance of four above-mentioned enzymes, studied in the course of this doctoral project, has been emphasized.

# Table of Contents

# Abbreviations

| | |
|---|---|
| aa | amino acid(s) |
| ADH | alcohol dehydrogenase |
| AlDH | aldehyde dehydrogenase |
| Amp | ampicillin |
| AMP | adenosine monophosphate |
| Arg | arginine |
| Asn | asparagine |
| Asp | aspartic acid |
| ATP | adenosine triphosphate |
| bp | base pair(s) |
| BRENDA | database compiling enzymes and enzyme classes, accessible under http://www.brenda-enzymes.org/ |
| CAR | carboxylic acid reductase |
| CoA | coenzyme A |
| Cys | cysteine |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DTT | 1,4-dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EC | enzyme class |
| EDTA | ethylenediaminetetraacetic acid |
| EMBL-EBI | European Bioinformatic Institute of European Molecular Biology Laboratory, accessible under: http://www.ebi.ac.uk/ |
| EVC | empty vector control |
| eAsA | D-erythroascorbic acid |
| FAD | flavin adenine dinucleotide |
| Fe-ADH | iron-dependent alcohol dehydrogenase |

| | |
|---|---|
| GDH | glucose dehydrogenase |
| HCl | hydrogen chloride |
| His | histidine |
| HLADH | horse liver alcohol dehydrogenase |
| HT/6xHis-tag | polyhistidine-tag consisting of six histidine residues |
| 3-HT | 3-hydroxytyrosol |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| IUBMB | International Union of Biochemistry and Molecular Biology |
| Kan | Kanamycin |
| kDa | kilodalton, 1kDa = 1 kg/mol |
| LB | lysogeny broth (tryptone: 10 g/L, yeast extract: 5 g/L, sodium chloride: 5 g/L)[1] |
| Lys | lysine |
| MDH | medium chain dehydrogenase |
| MS | mass spectrometer |
| MSM | minimal salt media |
| N/D | no data |
| NaCl | sodium chloride |
| NAD(H) | nicotinamide adenine dinucleotide (reduced form) |
| NADP(H) | nicotinamide acenine dinucleotide phosphate (reduced form) |
| NB | nutrient broth |
| NCBI | National Center for Biotechnology Information, accessible under: http://www.ncbi.nlm.nih.gov/ |
| *Ni*CAR | carboxylic acid reductase from *Nocadia iowensis* sp. NRRL 5646 |
| OD | optical density |
| ON | overnight |
| 5-*O*-TrR | 5-*O*-trityl-α,β-D-ribose |

[1] This composition of LB medium was used throughout whole thesis

| | |
|---|---|
| PCR | polymerase chain reaction |
| PDB | Protein Data Base |
| rpm | revolutions per minute |
| *Ras*AlDH | aldehyde dehydrogenase from *Ralstonia eutropha* H16 |
| *R. eutropha* | *Ralstonia eutropha* |
| RT | room temperature |
| SCOs | single-cell oils (edible cell oils extracted from microorganisms) |
| SCPs | single-cell protein (source of mixed protein extracted from pure or mixed cultures of algae, yeasts, fungi or bacteria (grown on agricultural wastes) used as a substitute for protein-rich foods, in human and animal feeds) |
| SDR | short chain dehydrogenase/reductase |
| Ser | serine |
| TNT | 2,4,6-trinitrotoluene |
| Tris | tris(hydroxymethyl)aminomethane, (2-amino-2-hydroxymethyl-propane-1,3-diol) |
| TSB | tryptic soy broth |
| Tyr | tyrosine |
| U | enzyme unite, 1U catalyzes the conversion of 1 µmole of substrate per minute |
| UniProtKB | Universal Protein Resource Knowledgebase, accessible under: http://www.uniprot.org/ |
| UV | ultraviolet light |
| *Y. lipolytica* | *Yarrowia lipolytica* |
| *Yl*SDR | shord chain dehydrogenase/reductase form *Yarrowia lipolytica* CLIB 122 |

# 1 Introduction

Enzymes play an important role in all living organisms, enabling them a proper functioning. Moreover, their application in food production accompanied people from the beginning of civilization. Initially unidentified, but responsible for rising of bread and for glucose fermentation, they have become an important part of human life [1]. Isolation of the first enzyme - α-amylase in 1833 by Anselme Payen [2] became a flash point for the development of a new branch of science – enzymology and, subsequently, biotechnology.

The Enzyme Commission, set up by the International Congress of Biochemistry in 1956, developed an enzyme nomenclature based on the chemical reactions catalyzed by them [3]. For identification purposes, every enzyme possesses a unique four-digit number, described as EC A.B.C.D, where:

A. shows to which of the six main divisions (classes) the enzyme belongs and indicates by that the main type of catalyzed reaction;
B. indicates the subclass, defining the substrate class or the type of the transferred molecules;
C. gives the sub-subclass, which indicates the co-substrate;
D. is the individual enzyme number in its sub-subclass.

Six enzyme classes encompass:

- EC 1 *Oxidoreductases*: catalyze redox reactions;
- EC 2 *Transferases*: transfer a functional group (for example a methyl, acyl or phosphate group) from one substrate to another;
- EC 3 *Hydrolases*: catalyze the hydrolysis of C-O, C-N, C-C and some other bonds;
- EC 4 *Lyases*: catalyze the cleavage of C-O, C-N, C-C and some other bonds by elimination;
- EC 5 *Isomerases*: catalyze isomerization changes within a single molecule;
- EC 6 *Ligases*: join two molecules with covalent bonds [4,5].

From these six classes, the oxidoreductases were the subject of these doctoral projects and therefore the further parts of this thesis will be dedicated to them.

## 1.1 Oxidoreductases

Oxidoreductases are widely distributed among all branches of the tree of life and catalyze the transfer of electrons or redox equivalents between donor and acceptor molecules [6]:

$$A^- + B \rightarrow A + B^-$$

where:
A – the reductant
B – the oxidant

According to the enzyme classification, oxidoreductases were divided into 24 subclasses [7]. For their industrial application they were classified by the type of catalyzed reaction and/or coenzyme-dependence into following groups [5,6]:

- Dehydrogenases/reductases (also called carbonyl reductases), (NAD(P)H – dependent);
- Oxygenases/hydroxylases (some NAD(P)H – dependent), using molecular oxygen as a cosubstrate;
- Oxidases (for example flavoproteins), catalyzing the electron-transfer onto molecular oxygen
- Peroxidases, requiring hydrogen peroxide or its chemical derivative.

Although in the BRENDA database, oxidoreductases constitute the majority (1694 records [8], they are only on the second place in industrial application, preceded by hydrolases (Figure 1) [9]. However, it is predicted that the demand for oxidoreductases will increase in the future [5,9].

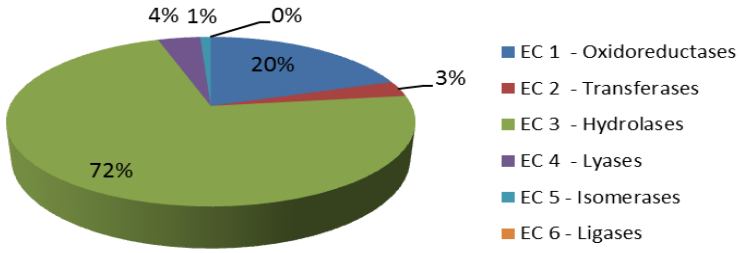


**Figure 1. Distribution of technical enzyme applications over IUBMB enzyme classes.** Adapted from Ref. [9].

Oxidoreductases are used in the industrial, food, environmental, biosensor and bioreporter medical and personal care as well as in organic synthesis fields. Especially in the latter, oxidoreductases have a great potential, because they are versatile and can be highly chemo-, regio-, stereo-, enantio- and enantiotoposelective. Since they can work at room temperature and in mild conditions, using renewable biomaterials to build synthons, they perfectly fit to the rising demand for environmental-friendly solutions [6].

This thesis was aimed at the discovery of versatile enzymes for transformations of particular substrates to industrially relevant products. A number of structurally distinct target reactions were investigated, and, in course of this project, twenty-five proteins were considered as potential biocatalysts, nineteen were successfully expressed under the chosen conditions and four enzymes were chosen for detailed studies:

- Alcohol dehydrogenase 2 from *Yarrowia lipolytica* CLIB 122 (*Yl*ADH2) [10], EC 1.1.1.1*;*
- Short chain dehydrogenase/reductase from *Yarrowia lipolytica* CLIB 122 (*Yl*SDR), putative mannitol-2-dehydrogenase, EC 1.1.1.138 [11];
- Aldehyde reductase from *Ralstonia eutropha* H16 (*Cupriavidus necator*), EC 1.1.1.21;
- Carboxylic acid reductase from *Nocardia iowensis* sp. NRRL 5646, EC 1.2.1.30 [12]

Therefore, a short introduction in the world of alcohol dehydrogenases will be provided now (carboxylic acid reductases are described in the further part of the thesis, on the page 62).

### 1.1.1 Classification of alcohol dehydrogenases

Alcohol dehydrogenases (ADHs) and short chain dehydrogenases/reductases (SDRs) belong to the same enzyme class EC 1.1.1 - oxidoreductases acting on the hydroxyl group of donors with $NAD(P)^+$ as an acceptor. In other words, they catalyze the reversible oxidation of primary or secondary alcohols by simultaneous reduction of $NAD(P)^+$ with a hydride [7].

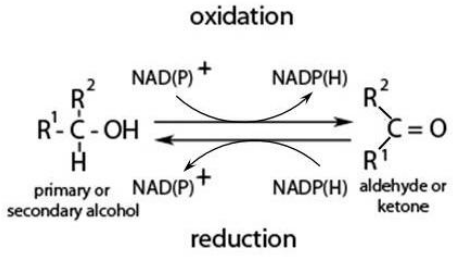


**Figure 2. Reversible reaction catalyzed by enzymes belonging to EC 1.1.1 subclass.** Adapted from Ref. [13].

Members of both superfamilies (SDRs and ADHs) are especially desired in pharmaceutical and agrochemical industries for producing chiral alcohols, which are in turn valuable building blocks in organic chemical synthesis [14]. SDRs and ADHs can be found in almost all living organisms, where they participate in many biochemical processes [13,15,16].

According to the classification proposed by Reid and Fewson in 1994, there are three categories of alcohol dehydrogenases [17]:

I. $NAD(P)^+$-dependent dehydrogenases;

II. $NAD(P)^+$-independent enzymes, using pyrroloquinoline quinone, haem or cofactor F420 as cofactors;

III. FAD-dependent alcohol oxidases.

The dehydrogenases, which are the subject of this thesis, belong to category one, the NAD(P)-dependent alcohol dehydrogenases, hence, just this category was characterized in more details in Table 1. SDRs are part of this category, which additional encompasses medium chain alcohol dehydrogenases (MDH) and iron-dependent alcohol dehydrogenases (Fe-ADH), (Table 1) [17]. The main difference between SDRs and MDHs superfamilies is the fact, that the SDRs, in contrast to the MDHs, do not require a metal in the active site [16]. The further differences, including variety in the amount of subunits and the number of amino acid residues as well as disparities in the active site were collected in Table 1, created on the basis of available sources [16–22].

**Table 1. Classification of microbial alcohol dehydrogenases [16–22]**

| Category | | Group No. | Superfamily | Chain length | No. and type of domains | Type of subunits assembly | Metal dependence | Catalytic centre |
|---|---|---|---|---|---|---|---|---|
| I | $NAD(P)^+$-dependent dehydro-genases | 1 | Long-chain (re-classified as medium chain) alcohol dehydrogenase (MDH) | ~350 aa | 2 (N-terminal catalytic and C-terminal $NAD(P)^+$ binding - Rossman fold) | Dimers Tetramers | Zn-dependent (two $Zn^{2+}$ atoms per subunit) | Catalytic triad of Cys, His and Cys |
| | | 2 | Short chain dehydrogenase/ reductase (SDR) | ~250 aa (sometimes ~350 aa, when the C-terminal region is extended) | 1 (Rossmann fold) | Monomers Dimers Tetramers | No metal dependence | Catalytic tetrad of Tyr, Lys, Ser and Asn |
| | | 3 | "iron-activated" or "metal-dependent polyol dehydrogenases [20]" | > 350 aa | 2 (N-terminal $NAD(P)^+$ binding with Rossman-fold like topology and C-terminal catalytic) [21] | Dimer [22] | $Fe^{2+}$ or $Zn^{2+}$-dependent | Catalytic tetrad of His, His, His and Asp |

### 1.1.2 Mechanism of cofactor binding

The common feature shared by MDHs and SDRs is the presence of Rossmann fold, which is one of the three most abundant folds in the Protein Data Bank (PDB) [23]. It consists of two sets of β-α-β-α-β units (Figure 3 A). These units form a single central twisted β-sheet of 6 or 7 parallel β-strands flanked by antiparallel α-helices on each side (Figure 3 B). The number of α-helices can be different for different proteins. The β-strands are arranged from left to right in the following order: (7)-6-5-4-1-2-3 with the long loop between strands 3 and 4, which creates enough cavity to bind the adenosine of NAD(P)(H) [24].

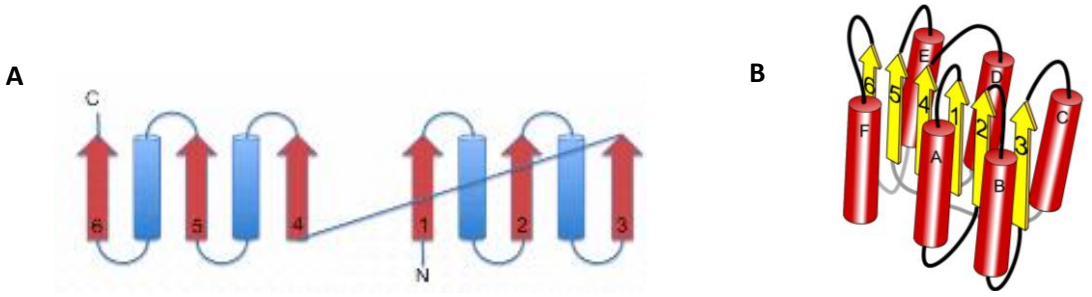


Figure 3. Topology of Rossmann fold. (A) Two sets of β-α-β-α-β units connected with a long loop between 3rd and 4th strand [25]; (B) Six α-helices flanking the central β-sheet consisting of 6 β strands in Rossmann fold of human SDR (PDB entry 1xg5) [26].

The binding of the cofactor involves numerous hydrogen-bonds and van der Waals' interactions between the cofactor and the enzyme. Especially strong are hydrogen bonds between the phosphate group of the cofactor and the turn between first β-strand and the subsequent α-helix of the Rossmann fold topology [24]. This turn exhibits a consensus binding pattern GX1-2GXXG [23] (TGXXX[AG]XG and its variation in case of SDRs [27]), in which the first two glycines participate in NAD(P)-binding, and the third facilitates close packing of the α-helix to the beta-strand [24]. Typically, the subsequent α-helix possess GXXXG or GXXXA motif, whereby the first glycine of this motif and the third glycine of the GX1-2GXXG motif are the same. These two motifs stabilize the Rossmann fold and it was proposed to combine them into one extended motif: V/IXGX1-2GXXGXXXG/A [23]. Additionally, the glycine or alanine of the GXXXG/A motif form van der Waals interactions with either valine or isoleucine located upstream of the GX1-2GXXG motif (Figure 5) [23]. In most of the dehydrogenases a conserved aspartate residue is located in a distance of about 20 residues C-terminal to the GXGXXG motif. This aspartate is responsible for the creation of hydrogen bonds with the ribose residue of the adenosine moiety of $NAD^+$, as it is shown in Figure 4 [24].

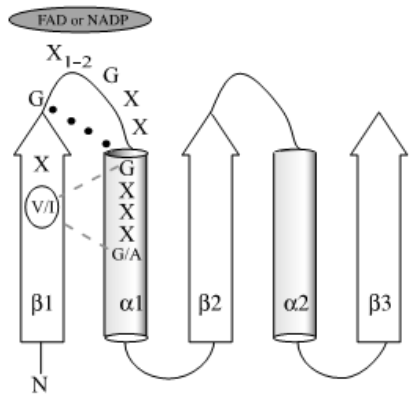


**Figure 5. Secondary structure and sequence motifs of the FAD or NAD(P)-binding Rossmann fold.** The loop contains $GX_{1-2}GXXG$ motif, which is followed by the GXXXG/A motif on the α-helix. Black-dotted line shows hydrogen bonds between the first and the last glycine residue in the $GX_{1-2}GXXG$ motif. Dashed lines show van der Waals' interaction between the glycine or alanine residues of the GXXXG/A motif located on the α-helix and the valine/isoleucine residue on the first β-strand. From Ref. [23].

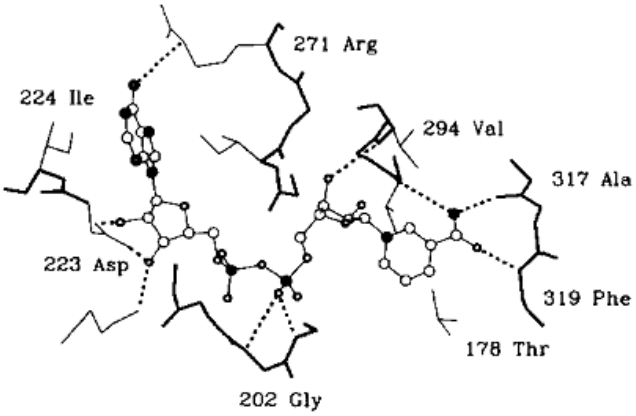


**Figure 4. Interaction between the $NAD^+$ cofactor (represented as ball-and-stick diagram) and NAD-binding domain in horse liver alcohol dehydrogenase.** From Ref. [24].

### 1.1.3 Catalytic mechanism of medium chain alcohol dehydrogenases

As it was already mentioned in Table 1, MDHs have two domains per protein subunit: one coenzyme-binding and one substrate-binding domain. Each of them binds one zinc ion: a structural and a catalytic one, thus two zinc atom per protein subunit [18]. During alcohol oxidation, the catalytic zinc atom serves for stabilization and orientation of the substrate, whereas during aldehyde

reduction the zinc atom polarizes the substrates by increasing its electrophilicity and making it attractive for the nucleophilic attack from the cofactor's hydride [17].

The in-depth studies of horse liver dehydrogenase showed that the catalytic zinc atom in MDHs is four-coordinated [28], although also five-coordination was reported [29]. However, it was proved that four-coordinated catalytic zinc is about 40 kJ/mol more stable in the enzyme than the five-coordinated one [30,31]. The active-site zinc is coordinated with two sulfur atoms from Cys46, Cys174 and a nitrogen atom from His67 (numbering is based on HLADH), which are conserved among all microbial ADHs [17,32]. The fourth position is occupied by an ionizable water molecule, which also interacts with the hydroxyl group of Thr48 [33]. After substrate binding, water is replaced by substrate, which in turn is tacked over the benzene ring of Phe93 (Figure 6 A) [28].

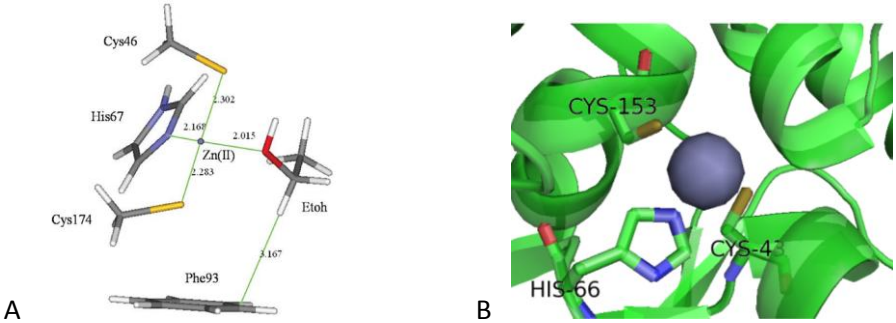


**Figure 6. Coordination of zinc atom (in grey). (A)** In the active site of HLADH with ethanol as a substrate. From Ref. [28]; **(B)** In the active site of *Sc*ADH1 (PDB: 2hcy) by His66, Cys 43 and Cys153. The image was generated with PyMol 1.1.3.

The catalytic mechanism of alcohol oxidation consists of the following steps:

1. Binding of $NAD(P)^+$ to the C-terminal coenzyme binding site of the Rossmann fold ($GX_{1-2}GXXG$) [23];
2. Binding of the alcohol substrate and thereby replacement of the zinc-bound water molecule in the active site;
3. Deprotonation of the alcohol substrate (alkoxide formation);
4. Transfer of the hydride ion from the alkoxide ion to the C4-position of the nicotinamide ring of the $NAD(P)^+$ (reduction of the cofactor and creation of zinc-bound aldehyde);
5. Release of the aldehyde and dissociation of NAD(P)H [30].

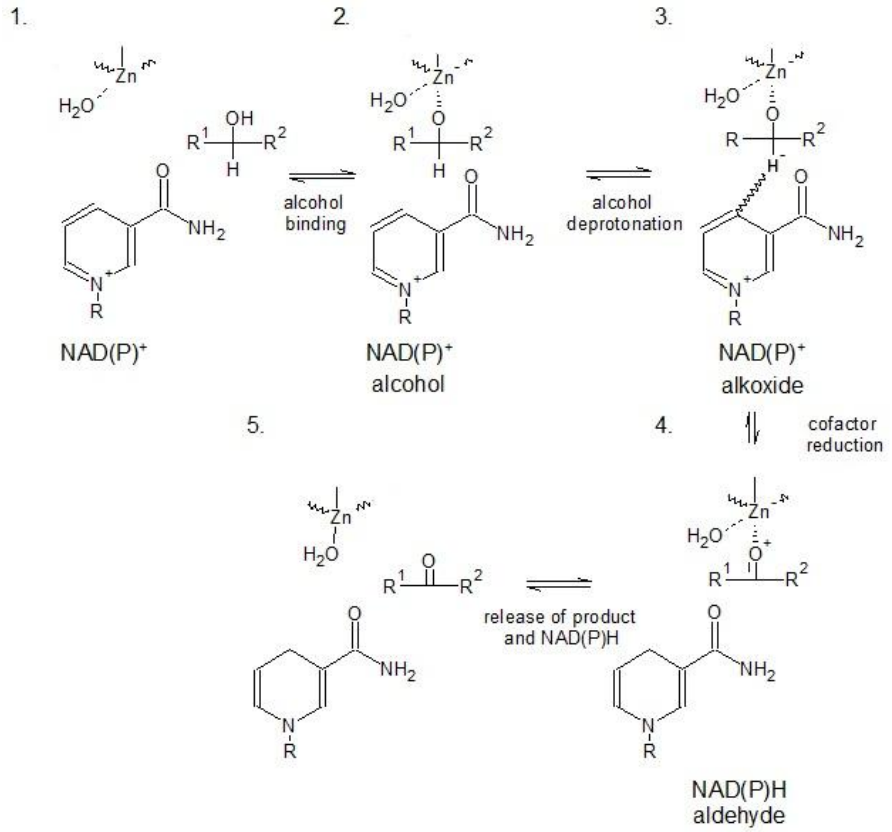


**Figure 7. Mechanism of action of alcohol dehydrogenase.** Adapted from ref [34].

Hydride transfer to and from the pro-chiral carbon at the pyridine C4-position of the nicotinamide ring of NAD(P)(H) catalyzed by dehydrogenases is always stereospecific: some dehydrogenases transfer the hydride ion to one side of the nicotinamide ring, whereas others – to the opposite side. Hence, the enzymes can be classified as:

- Class-A enzymes – binding the nicotinamide ring in the *anti* conformation and transferring the hydride ion from/to A-side of the ring (*re* face or *pro*-R position of NAD(P)(H));
- Class-B-enzymes – binding the nicotinamide ring in the *syn* conformation and transferring the hydride ion from/to B-side of the ring (*si* face or *pro*-S position of NAD(P)(H)) [35].

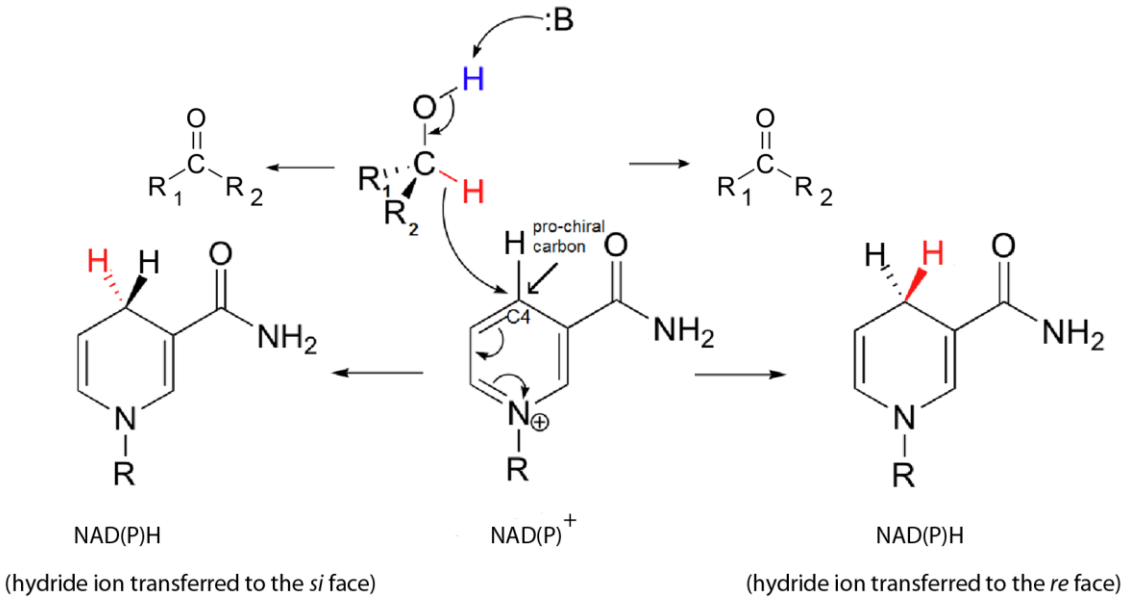


**Figure 8. Hydride transfer from an alcohol (primary or secondary) to the *si* or to the *re* face of the nicotinamide ring**. Adapted from ref [35].

Moreover, in the opposite reaction (hydrogenation) there is also a second type of alcohol dehydrogenase stereospecificity, namely, the stereospecificity of the obtained alcohol product. Similar to the cofactor, also in this case the hydride can attack from *re* or *si* face of a pro-chiral carbon, leading to the *S* or *R* alcohol formation, respectively. This type of enzyme stereospecificity is determined by the interaction between NAD(P)H and aldehyde/ketone substrate, and depends on which side of the cofactor the substrate is bound. Additionally, ADHs transfer either the *pro*-S or the *pro*-R hydrogen from the C4 of nicotinamide ring to the pro-chiral carbon of aldehyde/ketone (Figure 9) [35]. In the reverse reaction (dehydrogenation), ADHs stereospecifically catalyze the oxidaition of either *R* or *S* alcohol, what makes them so desired for kinetic resolution.

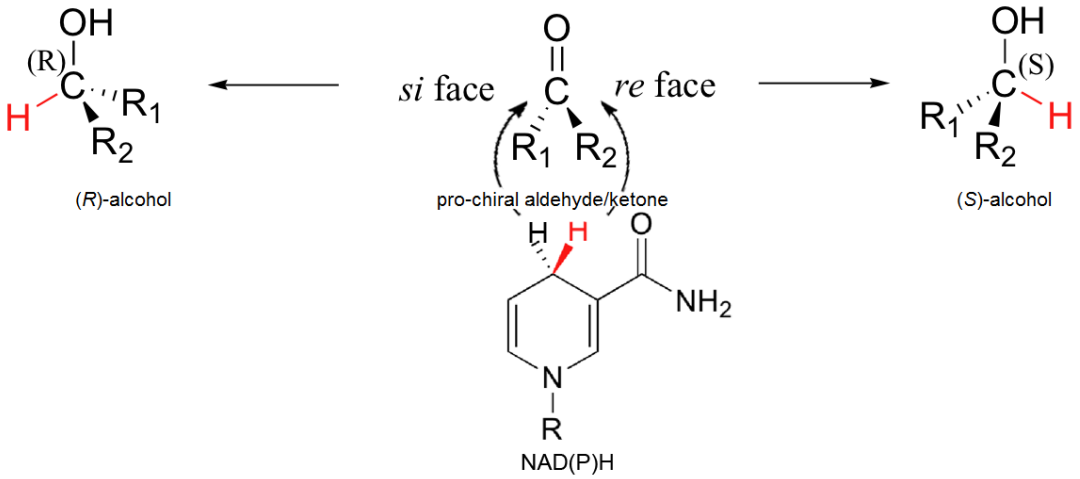


**Figure 9. Formation of (*R*)- or (*S*)- alcohol, depending on the direction of hydride attack on pro-chiral carbon.** Adapted from Ref. [35]**.**

### 1.1.4 Catalytic mechanism of short chain dehydrogenases/reductases

As it was already mentioned at the beginning of the section 1.1.1 (p. 13) the main difference between MDHs and SDRs is the absence of metal ion in the active center in these latter and the presence of a single domain in them [16]. Hence, although they catalyze common reactions, they work according to different mechanisms and thereby the catalytic centre of SDRs is different than this of ADHs. Namely, in most of the SDRs it consists of catalytic tetrad of asparagine-serine-tyrosine and lysine (Asn-Ser-Tyr-Lys), which, although the most common, it is not very conserved among SDRs [27]. In this catalytic tetrad Tyr (as the most conserved in the whole SDR superfamily) functions as the catalytic base. Lys lowers the $pK_a$ of Tyr hydroxyl group to promote proton transfer. Additionally, the lysine interacts with the ribose moiety of the nicotinamide cofactor, stabilizing its orientation within the cofactor binding site. The serine creates a hydrogen bond with the substrate and the asparagine interacts with the side chain of Lys via a water molecule, thereby stabilizing its position and enabling proton transfer between all molecules involved in the reduction reaction (Figure 10) [36]. The reaction relies on a "bi-bi" mechanism, with the cofactor binding at first and leaving the last:

1. Binding of NAD(P)(H) to the N-terminal coenzyme binding site of Rossmann fold (TGXXX[AG]XG in case of classical SDRs) [27] ;
2. Binding of the substrate (alcohol in the case of dehydrogenases, aldehydes or ketones in the case of reductases) followed by conformational changes in the substrate binding loop;
3. Closure of the active site cleft;
4. Release of the product and dissociation of coenzyme [37].

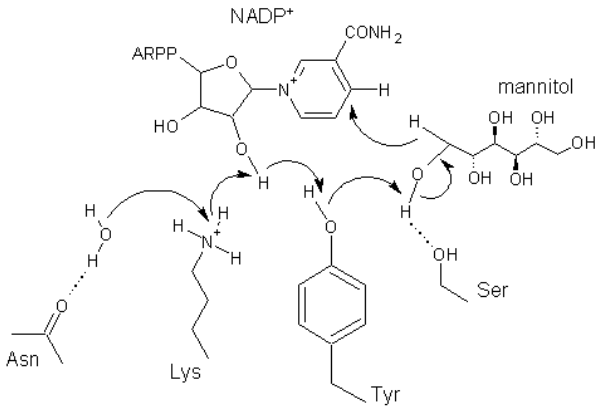


**Figure 10. Scheme of the mannitol reduction with catalytic tetrad of Tyr, Lys, Ser and Asn.** The proton transfer starts from water molecule present between the Asn and Lys. Adapted from Ref. [36].

For the cofactor preference among MDH and SDR superfamilies, the presence (or absence) of proximal Asp is responsible. In the NAD(H)-dependent SDRs the proximal Asp is present, because it interacts with O2 and O3 of the adenine ribose, but in NADP(H)-dependent enzymes it is absent, because otherwise it would cause an electrostatic repulsion against the negatively charged 2'-phosphate group of NADP(H). The proximal Asp in these enzymes is replaced by the presence of two positively charged amino acids: Lys and Arg, which, in contrast to Asp, interact with the 2'-phosphate group of nicotinamide [27,37]. However, it should be stressed that many other residues also contribute to coenzyme preference, hence more than one amino acid residue should be exchanged in order to completely reverse coenzyme specificity [27,38].

## 1.2 *Yarrowia lipolytica* and its enzymes

### 1.2.1 *Yarrowia lipolytica* – a non-conventional yeast

*Yarrowia lipolytica* is one of the ten non-conventional yeasts described in Wolf's handbook [39]. Since its maximum growth temperature seldom exceeds 34 °C, *Y. lipolytica* is considered as nonpathogenic. Originally isolated from dairy products and classified as *Candida lipolytica* in the early 1940s, it went through several reclassifications, from *Endomycopsis lipolytica*, to *Saccharomycopsis lipolytica* and finally, in 1980, it was classified as *Yarrowia lipolytica* by van der Walt and von Arx [40]. Because of its unconventional features, relying on the usage of *n*-paraffins (which were cheap and abundant in the 1960s) as a sole carbon source, it was intensively studied at that time. The ability to consume *n*-alkanes, 2,4,6-trinitrotoluene (TNT), isoprenoids and aromatic hydrocarbons (naphthalenes, phenathrene) by *Y. lipolytica* made it an interesting candidate for bioremediation processes of waste waters [41] and TNT-polluted marine environments [42]. Because of its lipolytic activity, this microorganism has also been applied in bioremediation of oil-contaminated environments, like olive mill waste water and palm oil mill effluents [41]. Moreover, its ability to produce high amount of single cell proteins (SCP), single cell oils (SCO), organic acids (2-ketoglutaric, pyruvic, isocitric and citric acid), γ-decalactone (a peach-like aroma compound) and biosurfactants from by-products of biodiesel synthesis made from *Y. lipolytica* an industrial workhorse [41,43].

Evolutionary analysis of genomes of four yeast species from the hemiascomycetes (saccharomycetes) class, done by Génolevures project members, placed *Y. lipolytica* on an isolated branch of the phylogenetic tree (Figure 11). This genome-sequencing program was devoted to genomic analysis of different hemiascomycetous yeasts species, among which also *Y. lipolytica* CLIB 122 has been found [44]. During the investigation of *Y. lipolytica* it was discovered that it has a larger genome size (20.5 Mb), higher G+C content (49%) and lower gene density (1 gene per 3 kb) than other analyzed yeast species [45]; this could explain its unique features.

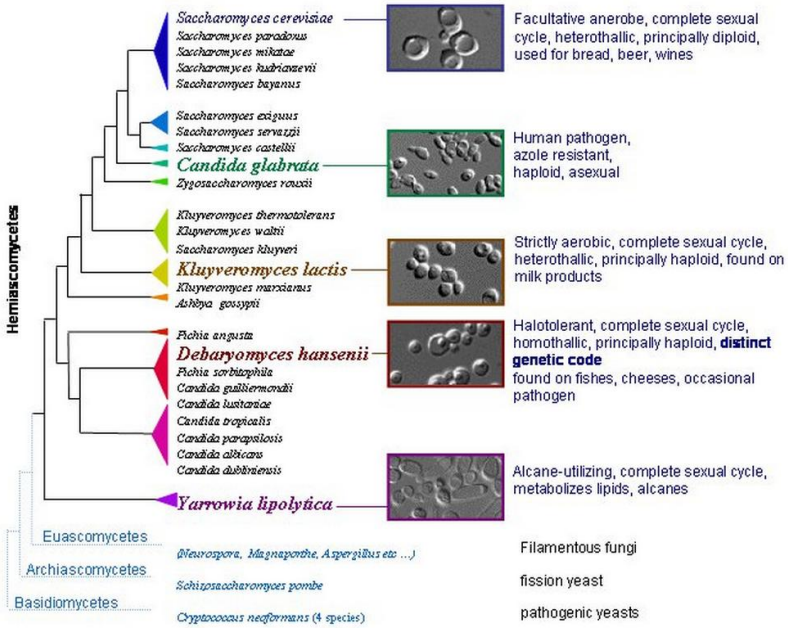


**Figure 11. Phylogenetic tree of four yeast species belonging to hemiascomycetes class.** From Ref. [44].

### 1.2.2 Enzymes from *Yarrowia lipolytica*

Although *Y. lipolytica* is mostly used on industrial scale for whole cell bioconversion [39], the UniProtKB database contains 442 enzymes from *Y. lipolytica*, mostly transferases and hydrolases (Figure 12) [46]. Among them, lipases are the most important metabolic products, because they are applied in the detergent, food, pharmaceutical and environmental industry [41]. Oxidoreductases (88, 44 reviewed) are on the third place of this list and constitutes 20% of all *Y. lipolytica's* enzymes (Figure 12).

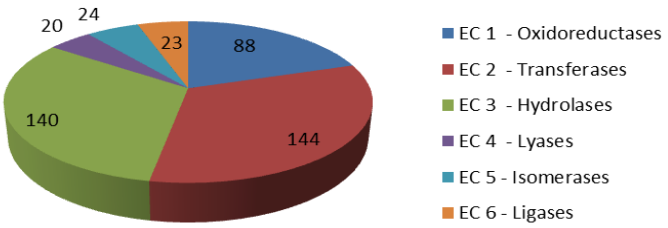


**Figure 12. Distribution of *Y. lipolytica* enzymes over IUBMB enzyme classes in UniProtKB [46].**

Among *Y.lipolytica's* oxidoreductases the most characterized by now are:

- Isocitrate dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42) [47];
- Short chain dehydrogenase/reductase (putative mannitol 2-dehydrogenase $NADP^+$, EC 1.1.1.138) [11];
- Alcohol dehydrogenases (EC 1.1.1.1) [10,48,49];
- Long-chain-alcohol dehydrogenase (EC 1.1.1.192) [34,50];
- Long-chain-alcohol oxidase (EC 1.1.3.20) [51];
- Long-chain-aldehyde dehydrogenases (EC 1.2.1.48) [34,52,53];
- Malate dehydrogenase (EC 1.1.1.40) [54];
- Phosphatidylcholine desaturase (EC 1.3.1.35) [55];
- Acyl-CoA oxidase (EC 1.3.3.6) [56,57];
- NADPH-hemoprotein reductase (EC 1.6.2.4) [58];
- NADH:ubiquinone oxidoreductases (EC 1.6.5.3, EC 1.6.5.8, EC 1.6.5.9) [59,60].

Since just ADHs and SDRs of *Y. lipolytica* are the main subject of this thesis, the following section will focus on them.

### 1.2.3 *Yarrowia lipolytica*'s medium chain alcohol dehydrogenases

The first attempt to characterize alcohol dehydrogenases of *Yarrowia lipolytica* (medium-chain length) was made by Barth and Künkel in 1979 [48]. On the basis of a native gel-electrophoresis they detected four different ADHs:

1. ADH I – $NAD^+$-dependent, with the mass of 240 kDa (in the discussion, the authors did not exclude existing of two $NAD^+$-dependent enzymes with the same electrophoretic mobility. This thesis was upheld also in their later work [43]);
2. ADH II, III and IV – $NADP^+$-dependent, with the mass of 120, 70 and 70 kDa, respectively.

The numbering of detected ADHs (I, II, etc.) based on the order of the bands' appearance on the gel (the band of ADH I as the biggest was the first) [48].

Although the authors published a number of other papers about *Y. lipolytica,* none of their work concerned alcohol dehydrogenases [39,43,61,62] Only in 1999, when the Génolevure's project has started, the ADH from *Y. lipolytica* became subject of scientific interest again. In the progress of genome characterization of the CLIB 122 strain, eleven alcohol dehydrogenases were found: ten medium chain ADHs having about 350 aa and possessing a catalytic Zn-binding site (Table 2) and one long chain ADH [45], (for clarity not included in Table 2). Additionally, Kim J. and Kim J.Y. isolated three MDHs from *Y. lipolytica* CX161-1B [63]. These enzymes, whose sequences can be found in the NCBI database, were classified as putative alcohol dehydrogenase 1, 2 and 3, respectively [64], but their deeper characterization is not available. Nevertheless, their homologs were identified also in CLIB 122 strain and the analysis of homologous MDHs revealed that their sequences are quite conserved:

- ADH1 from the strain CX161-1B differs from ADH1 from the strain CLIB 122 only in one aa: it has a glycine on position 255 instead of an asparagine;
- ADHs2 share 100% identity between these two strains;
- ADH3 from the strain CX161-1B has, in comparison to ADH3 identified in CLIB 122, just two additional aa: glutamic acid and leucine on the position 215 and 216, respectively.

Although the existence of ten MDHs was predicted in *Y. lipolytica* CLIB 122 (seven of them as putative) [45], to my knowledge just two of them were isolated and characterized by now in more detail:

- *Yl*ADH2 (YALI0E17787p), (Table 2, entry 2), whose kinetic characterization is part of this doctoral dissertation (section 3, p. 38) [10];
- Putative ADH, similar to ADH SFA from *S. cerevisiae*, whose isolation and a rough characterization can be found in Matatiele's dissertation [34].

This means that the MDHs from *Y. lipolytica* are still waiting for their exploration and can surprise us in the future with some extraordinary features, like the enzymes from an unconventional yeast should do.

By now the following conclusions can be drawn from analysis of amino acid sequences of identified ADHs: the multiple sequence alignment of ten MDHs identified in the *Y. lipolytica* CLIB 122 genome, done with Clustal Omega [65], revealed that the most similar are ADH1, 2 and 3 (they share more than 90% sequence identity, which may indicate that they are isoenzymes). Additionally, it can be observed, that ADH3 is more similar to ADH1 than to ADH2. YALI0A15147p shares around 80% identity with them, whereas YALI0E07766p only around 55% (Table 3). ADH2 is in turn the most similar to *Sc*ADH1 – a well-known Zn-dependent enzyme with known crystal structure (PDB: 2hcy), and therefore it was chosen for deeper characterization in the course of this thesis (p. 38, [10]). YALI0F09603p is the longest among the analyzed ADHs (381 aa), and share with other ADHs from 17.5% (with YALI0F08129p) until 27% identity (with ADH1), (Table 3). YALI0B10175p, YALI0F08129p, YALI0D02167p and YALI0E07810p shared between 52.8% to almost 65% identity among each other, but only about 17% with YALI0F09603p and about 25% with the others (Table 3).

**Table 2. Summary of medium chain alcohol dehydrogenases *of Yarrowia lipolytica* CLIB 122**

| Name | Gene ID | GeneBank No. | NCBI Ref. seq. | UniProt No. | Length [aa] | Mass [kDa] | Type of subunits assembly | Cofactor dependence |
|---|---|---|---|---|---|---|---|---|
| YALI0D25630p ADH1 | 2911068 | CAG81486.1 | XP_503282.1 | Q6C7T0 | 349 | 37.1 | homotetramer | NAD(H) |
| YALI0E17787p ADH2, similar to ADH1 from *S.cerevisiae* UniProt: P00330, PDB: 2hcy | 2912417 | CAG79670.1 | XP_504077 | F2Z678 | 351 | 37.3 | homotetramer | NAD(H) |
| YALI0A16379p ADH3 | 2906568 | CAG84058.1 | XP_500127.1 | Q6CGT5 | 349 | 37.1 | homotetramer | NAD(H) |
| YALI0A15147p Putative ADH, similar to ADH3 UniProt: Q9UW06 | 2905743 | CAG84018.1 | XP_500087.1 | Q6CGX5 | 348 | 37 | homotetramer | NAD(H) |
| YALI0E07766p Putative ADH, similar to mitochondrial YADH-3 from *S.cerevisiae* UniProt: P07246 | 2912456 | CAG79261.2 | XP_503672.2 | Q6C6P0 | 354 | 37.6 | homotetramer | NAD(H) |
| YALI0F09603p Putative ADH, similar to ADH SFA from *S.cerevisiae* UniProt: P32771 | 2908970 | CAG78022.1 | XP_505215.1 | Q6C297 | 381 | 40 | homodimer | NAD(H) |
| YALI0D02167p, Putative ADH, similar to ADH5 from *S.cerevisiae* UniProt: P38113 | 2910516 | CAG80501.1 | XP_502315.1 | Q6CAJ7 | 346 | 37.3 | N/D | NAD(P)(H) |
| YALI0F08129p Putative ADH, weakly similar to protein YIM1 from *S.cerevisiae* UniProt: P28625 and to oxidoreductase from *E. faecalis* UniProt: Q839Q1 | 2908523 | CAG77956.1 | XP_505149.1 | Q6C2G3 | 340 | 36.4 | N/D | NAD(P)(H) |
| YALI0E07810p Putative ADH, weakly similar to oxidoreductases from *L.plantarum* UniProt: Q890E1 | 2912458 | CAG79263.1 | XP_503674.1 | Q6C6N8 | 346 | 37.4 | N/D | NAD(P)(H) |
| YALI0B10175p Putative ADH, similar to oxidoreductase from *E. faecalis* UniProt: Q839Q1 | 2906782 | CAG82953.1 | XP_500708.1 | Q6CF54 | 341 | 36.8 | N/D | NAD(P)(H) |

**Table 3. Percent identity matrix of medium chain alcohol dehydrogenases of *Y. lipolytica* CLIB 122.** Matrix created by Clustal Omega 2.1 [65]. All values were rounded to full numbers and they are given in [%],

| | **YALI0B10175** | **YALI0F08129** | **YALI0D02167** | **YALI0E07810** | **YALI0F09603** | **YALI0E07766** | **YALI0A15147** | **YALI0E17787** | **YALI0D25630** | **YALI0A16379** |
|---|---|---|---|---|---|---|---|---|---|---|
| YALI0B10175 | 100 | 58 | 53 | 56 | 18 | 23 | 23 | 24 | 24 | 24 |
| YALI0F08129 | | 100 | 59 | 61 | 18 | 22 | 22 | 24 | 23 | 23 |
| YALI0D02167 | | | 100 | 65 | 19 | 22 | 21 | 24 | 24 | 23 |
| YALI0E07810 | | | | 100 | 18 | 22 | 23 | 24 | 25 | 25 |
| YALI0F09603 | | | | | 100 | 25 | 27 | 26 | 26 | 27 |
| YALI0E07766 | | | | | | 100 | 54 | 56 | 58 | 59 |
| YALI0A15147 | | | | | | | 100 | 78 | 81 | 82 |
| YALI0E17787 | | | | | | | | 100 | 93 | 93 |
| YALI0D25630 | | | | | | | | | 100 | 98 |
| YALI0A16379 | | | | | | | | | | 100 |

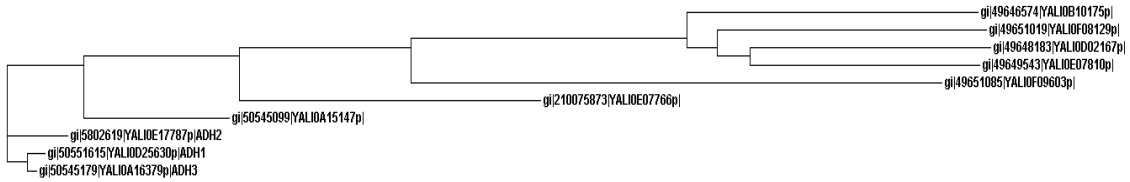


**Figure 13. Phylogram of medium chain alcohol dehydrogenases of *Yarrowia lipolytica***. The image was generated with Clustal Omega (1.2.0) [65].

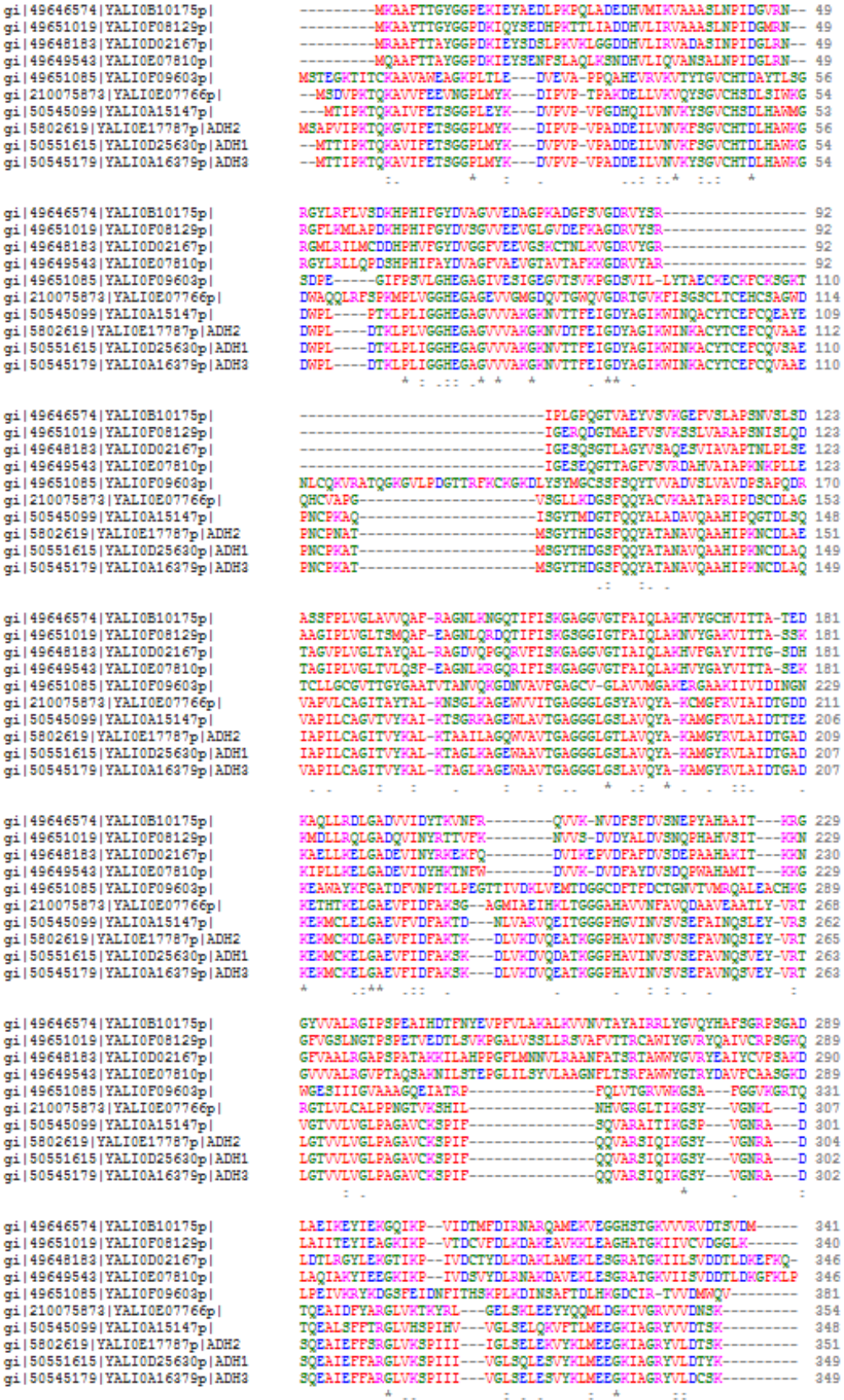

**Figure 14. Multiple sequence alignment of medium chain alcohol dehydrogenases of *Yarrowia lipolytica*.** The image was generated with Clustal Omega (1.2.0) [65]

### 1.2.4 *Yarrowia lipolytica*'s short chain dehydrogenase/reductases

In the NCBI database, 75 short chain reductase sequences of *Y. lipolitica* CLIB 122 enzymes are filed. Most of them belong to the short chain dehydrogenases/reductase family. Because of that, we would focus rather on these SDRs, which were characterized in more detail or found some commercial usage. Among such SDRs there are:

- *Yl*SDR (YALI0B16192p), EC 1.1.1.138 – putative mannitol dehydrogenase, whose kinetic characterization is also reported as part of this thesis (section 2, p. 27) and which was able to oxidize 5-*O*-trityl-α,β-D-ribose to the corresponding lactone [11];

- 3-ketodihydrosphingosine reductase TSC10 (YALI0B17688p), EC 1.1.1.102, catalyzes the reduction of 3-ketodihydrosphingosine to dihydrosphingosine, which, in turn, can be used for synthesis of sphingosine – an important component of food, pharmaceutical and cosmetic products [66];

- very-long-chain 3-oxoacyl-CoA reductase (YALI0A06787g), EC 1.1.1.330, which is a component of the microsomal membrane bound fatty acid elongation system and reduces 3-ketoacyl-CoA intermediate that is formed in each cycle of fatty acid elongation [67]. This feature was used for the production of very-long chain fatty acids in the recombinant *Y. lipolytica* strain [68].

From above examples it seems that short chain dehydrogenases/reductases from *Y. lipolytica* are unexplored to the same extent as medium chain dehydroganses from this organism. However, unique features of *Y. lipolytica* and its enzymes found by now, like growing of *n*-alkanes and oxidation of substrates with bulky protecting groups, make them (both the whole microorganism as well as its enzymes) very encouraging for further investigation. Examples of them can be already found on the following pages: 27and 38.

# 2 *Yarrowia lipolytica* dehydrogenase/reductase

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## *Yarrowia lipolytica* dehydrogenase/reductase: An enzyme tolerant for lipophilic compounds and carbohydrate substrates

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ABSTRACT:

*Yarrowia lipolytica* short chain dehydrogenase/reductase (*Yl*SDR) was expressed in *Escherichia coli*, purified and characterized *in vitro*. The substrate scope for *Yl*SDR mediated oxidation was investigated with alcohols and unprotected carbohydrates spectrophotometrically, revealing a preference for secondary compared to primary alcohols. In reduction direction, *Yl*SDR was highly active on ribulose and fructose suggesting that the enzyme is a mannitol-2-dehydrogenase. In order to explore substrate tolerance especially for space-demanding, lipophilic protecting groups, 5-*O*-trityl-ribitol and 5-*O*-trityl-α,β-D-ribose were investigated as substrates: *Yl*SDR oxidized 5-*O*-trityl-ribitol and 5-*O*-trityl- α,β -D-ribose and reduced the latter at the expense of NADP(H).



Oxidation reactions in general and selective oxidation reactions on carbohydrates in particular are very important chemical transformations and are frequently carried out using toxic (chromate based or sulfide releasing), expensive (Dess-Martin) and often unselective reagents.[1] To circumvent these typically harsh and uneconomic reaction conditions of chemical oxidation we were interested to find an enzyme with the ability to oxidize polar as well as non-polar carbohydrate compounds. *Yarrowia lipolytica* is a non-conventional yeast that is often found in lipid-rich media[2] and its machinery of enzymes seems to be well developed to metabolize both polar and non-polar substrates.[3] This prompted us to investigate enzymes from this organism.

In a recent publication, 5-chloro-5-deoxy-α,β-D-ribose was oxidized to the respective 5-chloro-5-deoxy-ribonolactone by the short chain dehydrogenase/ reductase (SDR) SalM from the marine organism *Salinispora tropica*.[4] We amplified the gene of a homologous short chain dehydrogenase (NCIB Accession No XM_500963.1, 30% identity to SalM) from genomic DNA of the *Yarrowia lipolytica* CLIB122 strain and cloned the gene into the pK470 vector,[5] introducing also an N-terminal His-tag to facilitate protein purification. The construct was transformed into *E. coli* BL21 (DE3) Gold. The strain was cultivated in Luria Broth medium, *Yl*SDR expression induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and supplemented with 1% glucose at $OD_{600}$ 0.6-0.8 and the expression phase carried out for 16-18 h hours at 28 °C. Collected cells were frozen at -20°C for whole cell biotransformations or sonicated and the *Yl*SDR protein purified by Ni-affinity chromatography over a GE Healthcare HisTrap FF column according to the manufacturers protocol. The eluate was re-buffered into 100 mM TrisHCl, pH 10.5 containing 50 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP) and 1% v/v glycerol, the protein concentrated by spin column centrifugation and aliquots were stored at -80 °C.

First, in order to explore the substrate scope of *Yl*SDR, several commercially available compounds such as simple primary and secondary alcohols of different chain length, selected aldoses, ketoses and polyols as well as two aldehydes were tested. Whereas for SalM the preferred cofactor is $NAD^+$,[4] it was $NADP^+$ for *Yl*SDR.[6] Its closest homologue for which a crystal structure is

known (*Candida parapsilosis* carbonyl reductase, pdb: 3ctm) was also reported to be NADP(H) dependent.[7]

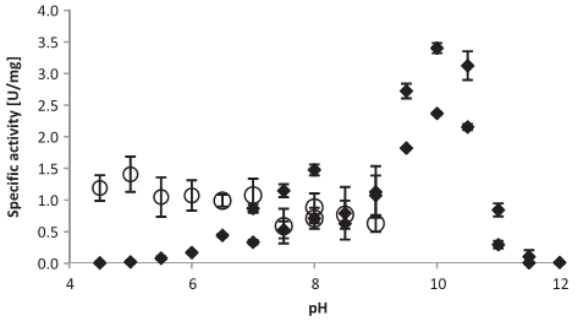



**Figure 1.** pH optima of *Yl*SDR. pH 4.5-6.0: 50mM citrate buffer, pH 6.0-8.0 and 10.5-12.0: 50mM potassium phosphate buffer, pH 8.0-10.5: 50mM Tris-HCl buffer; ♦oxidation of *rac*-2-heptanol; o reduction of fructose.

**Table 1.** Exploration of substrate spectrum of *Yl*SDR

| Entry | Substrate | Relative oxidation activity (%) | Substrate | Relative reduction activity (%) |
|---|---|---|---|---|
| 1 | *rac*-Phenylethanol | 28 | Phenylacet-aldehyde[b] | 16 |
| 2 | *rac*-1-Phenyl-1,2-Ethanediol | 51 | Acetophenone | 0 |
| 3 | 2-Propanol | 3 | Acetone | 0 |
| 4 | (2R,3R)-Butanediol | 3 | | |
| 5 | *rac*-4-Methyl-2-Pentanol | 6 | | |
| 6 | Cyclohexanol | 8 | Cyclohexanone | 0 |
| 7 | *rac*-2-Heptanol | 100[a] | 2-Heptanone | 19 |
| 8 | 1-Octanol | 0 | Octanal | 0 |
| 9 | *rac*-2-Octanol | 70 | 2-Octanone | 16 |
| 10 | (*R*)-2-Octanol | 97 | | |
| 11 | (*S*)-2-Octanol | 36 | | |
| 12 | octanal | 7 | | |
| 13 | 1-Nonanol | 5 | | |
| 14 | 2-Nonanol | 27 | | |
| 15 | 1-Necanol | 3 | | |
| 16 | 1-Dodecanol[c] | 4 | | |
| 17 | Ribitol | 0 | Ribulose[d] | 100 |
| 18 | Arabitol | 18 | Arabinose | 0 |
| 19 | Xylitol | 9 | Xylose | 0 |
| 20 | Sorbitol | 24 | Glucose | 0 |
| 21 | Mannitol | 55 | Fructose[d] | 33 |

[a] The specific activity for the oxidation of *rac* 2-heptanol was 1.4±0.4 U $mg^{-1}$.
[b] TritonX 100 was used as solubilizer in 0.5% end concentration
[c] DMSO was used as co-solvent in 5% end concentration. 5% DMSO reduced the activity towards rac-2-octanol oxidation by 25%.
[d] Similar to other aldoses, ribose and mannose were not reduced

To explore enzyme characteristics and the substrate scope of *Yl*SDR, the consumption of $NADP^+$ was monitored at 340nm.[6] Substrate oxidation was tested between 22 and 38 °C and *Yl*SDR showed the highest activity between 25 and 28 °C. The optimal pH for the reaction appears to be 10.5 (Figure 1), however, further increase resulted in a drastic deactivation. Whereas polyols such as xylitol or D-mannitol were readily oxidized according to the photometric measurements, sugars such as D-xylose, D-glucose or D-mannose were not. In fact, secondary alcohols were preferred: for the tested compounds, *Yl*SDR showed the highest activity for racemic 2-heptanol (1.34 ± 0.44 U/mg), followed by racemic 2-octanol. Although the enzyme had higher activity for (*R*)-2-octanol, also its (*S*) enantiomer was oxidized (Table 1).

Furthermore, *Yl*SDR turned out to be an efficient reductase for selected substrates: whereas 2-heptanone and 2-octanone were reduced with similar efficiency to the oxidation of the corresponding alcohols (0.94 ± 0.24 and 0.81 ± 0.38 U/mg), specific activities for fructose and ribulose reduction were 1.65 ± 0.47 and 4.96 ± 0.57 U/mg.[8] Since aldopyranoses were not reduced within the observed timeframe, *Yl*SDR may be a member of the NADP(H) dependent mannitol-2-dehydrogenase family (EC1.1.1.138), as also suggested by its sequence similarity to other proteins of this family.[7,9]

**Table 2.** Apparent kinetic parameters for *Yl*SDR

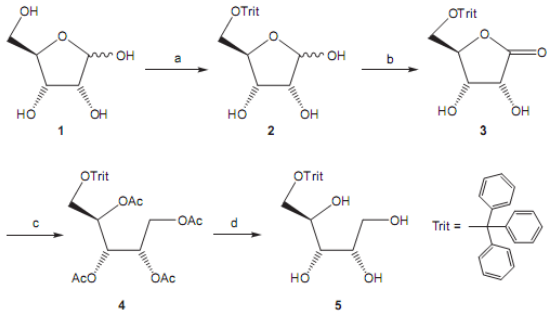
| | **Oxidation** | | **Reduction** |
|---|---|---|---|
| | ***rac*-2-heptanol** | **$NADP^+$** | **ribulose** |
| $K_m$ [mM] | 4.91 ± 0.31 | 0.79 ± 0.03 | 8.80 ± 2.10 |
| $k_{cat}$ [$s^{-1}$] | 3.17 ± 0.15 | 6.15 ± 0.28 | 5.42 ± 0.14 |
| $k_{cat}/K_m$ [$s^{-1}mM^{-1}$] | 0.65 ± 0.08 | 7.75 ± 0.06 | 0.62 ± 0.24 |

The determination of kinetic parameters for oxidation and reduction corroborated the different specific activites for oxidation and reduction, showing that the $k_{cat}$ value for reduction of ribulose is significantly higher than for oxidation of racemic 2-heptanol (Table 2). Whereas oxidations appeared most efficient at pH 10.5, reductions may be carried out between pH 4.5-10 (Figure 1). This remarkably broad operational stability for a wild type enzyme renders it a good candidate for industrial applicability.

In parallel, we investigated partly protected carbohydrates as substrates for this enzyme. The results for SalM driven oxidation of 5-chloro-5-deoxy-α,β-D-ribose[4], prompted us to investigate 5-*O*-trityl-D-ribitol (**5**) and 5-*O*-trityl-α,β-D-ribose (**2**) as model substrates. We reasoned that trityl ethers should exhibit better compatibility with biocatalytic processes than, e.g., typical ester or amide protective groups which would be cleaved by omnipresent esterase or amidase/peptidase activities in whole cell biocatalysts. The trityl group is, however, space demanding (Scheme 2) and lipophilic, and therefore literature reports on the use of tritylated substrates in biocatalytic steps are scarce and limited to lipase catalyzed esterifications.[10]

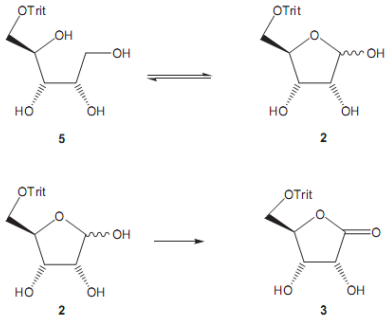
For the synthesis of substrates **2** and **5** conventional carbohydrate chemistry was applied. Starting from D-ribose, the primary hydroxy group at position C-5 underwent regioselective ether formation employing triphenylmethyl chloride in pyridine at 50 °C.[11] Instead of straight forward reduction of 5-*O*-trityl-α,β-D-ribose (**2**) to ribitol derivative **5**, aldose **2** was oxidized at the anomeric position employing bromine and $BaCO_3$ in water[12] to the corresponding 5-*O*-trityl-D-ribono-1,4-lactone (**3**), as this compound was needed for investigating biotransformation reactions of *Yl*SDR. The 1,4-lactone **3** was reduced employing $NaBH_4$ in

methanol and a per-*O*-acetylation step appeared to be necessary for purification reasons. Finally, 5-*O*-trityl-ribitol **5** was obtained by deacetylation under Zemplen conditions in very pure form.[13]




**Scheme 1.** Synthesis of 5-*O*-trityl-D-ribitol **5**: (a) Pyr, TritylCl, 50 °C, 50%; (b) $Br_2$, $BaCO_3$, $H_2O$, 45%; (c) 1) $NaBH_4$, MeOH; 2) $Ac_2O$, Pyr, rt 54%; (d) NaOMe 1 M, MeOH, rt, 86%.

In the following, 5-*O*-trityl-α,β-D-ribose (**2**) was used as a substrate for oxidative whole cell biotransformation reactions.[14] According to HPLC/MS, *Yl*SDR mediated oxidation occurred $NADP^+$ dependent at the C-1 position and gave 97% conversion of **2** to **3**. The product peak showed identical retention time and mass spectrum as the authentic standard (**5**) prepared by chemical means and was in pH-dependent equilibrium with the respective open chain acid. The whole cell biotransformation result was confirmed by reaction with purified *Yl*SDR,[15] yielding 96% **3**. Subsequently, 5-*O*-trityl-ribitol (**5**) was subjected to oxidation with whole cells and purified enzyme, which resulted in equally low conversion to mixtures of <5% **2** and approximately 10% of **3**. Whereas *Yl*SDR was unable to reduce **3**, **2** was partly reduced to **5**. However, in course of the reduction, $NADP^+$ is produced, which is consumed for the formation of **3**. Due to a reversible step (interconversion of **5** and **2**) in combination with an irreversible step (oxidation to **3**), the resultant mixture of products typically ends in approximately 10% **5**, 20% **2** and 70% **3** in reactions with purified enzyme, after overnight incubation.[14]




**Scheme 2.**Oxidation of 5-*O*-trityl-D-ribitol (**5**) and 5-*O*-trityl-α,β-D-ribose (**2**).

These findings seem to be in contradiction to the spectrophotometric results discussed above, in which neither ribitol nor ribose gave significant activities. However, it needs to be considered that typical photometric assays are restricted to initial rate measurements. Due to short monitoring times, interesting activities may easily be overlooked. We therefore monitored the oxidation of ribose for 12 h at pH 10.5 and found a specific activity of 10 mU/mg, admittedly much lower than those for other substrates (see Table 2) but certainly a useful starting point for protein engineering.

Summarizing, we have shown that *Yl*SDR is a versatile enzyme that catalyzes oxidation and reduction of polar as well as non-polar substrates at a very broad pH range. Secondary alcohols are preferred in the oxidation reaction compared to primary alcohols and aldehydes. Substrate selectivity was found for the reduction of medium chain length ketones with the carbonyl function at position C-2. In case of the carbohydrate substrates, alditols are preferred over aldoses as substrates in the oxidizing mode and ketoses (2-uloses) are accepted for the reduction step whereas aldoses are not. Most interestingly, a slightly different picture was obtained with 5-*O*-trityl-D-ribitol, -ribose and -ribonolactone. In the oxidation step 5-*O*-trityl-D-ribose (**3**) turned out to be a far better substrate than the corresponding ribitol **5**. In the reduction the same compound is a substrate whereas the lactone is not. In the light of these results, we are planning to explore the substrate scope of *Yl*SDR biotransformations in a structure activity relationship study by investigating further tritylated sugar substrates such as 5-*O*-trityl-D-mannitol and 6-*O*-trityl-D-fructose. Furthermore, different aromatic substituents at the terminal position of the carbohydrate moieties such as various nitro phenyl groups will be included to this study. In addition, *Yl*SDR mediated reductions of aldoses may be explored in the presence of for example NADPH oxidases to prevent shunt oxidation to the corresponding lactones.

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## Supplementary data

Supplementary data associated with this article can be found in the online version, at:

http://dx.doi.org/10.1016/j.bmcl.2013.03.064

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6. Unless otherwise stated, purified tagged *Yl*SDR (1-10 µM) was assayed in 50 mM Tris-HCl containing 2 mM $MgCl_2$, pH 8.0, 10 mM substrate (for lipophilic substrates, additional 0.15% Tween 20) and 1 mM $NADP^+$ or $NAD^+$. The increase of absorbance at 340 nm was monitored at 28 °C for 10 min (or 12 h for ribose). For $NAD^+$, no activity was observed as cofactor under these conditions. The reported values represent the average of at least four measurements with appropriate blanks substracted. One activity unit is defined as the amount of enzyme catalyzing the reduction of 1 µM of $NADP^+$ per minute. Kinetic parameters for oxidation were determined at pH 10.5 from unweighted non-linear least-square fits of experimental data using the program Sigmaplot (version 12.3).
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8. Unless otherwise stated, purified tagged *Yl*SDR (1-10 µM) was assayed in 50 mM Tris-HCl containing 2 mM $MgCl_2$, pH 8.0, 10 mM substrate (for lipophilic substrates, additional 0.15% Tween 20) and 0.75 mM NADPH. The decrease of absorbance at 340 nm was monitored at 28 °C for 10 min. The reported values represent the average of at least four measurements with appropriate blanks substracted. One activity unit is defined as the amount of enzyme catalyzing the oxidation of 1 µM of NADPH per minute. Kinetic parameters for reduction were determined at pH 5.0.
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13. Jung, M. E.; Xu, Y. *Tetrahedron Lett.* **1997**, *38*, 4199.
14. Typically, 100 mg of thawed whole cells were dispersed in 50 mM Tris-HCl buffer (pH 8.0 for oxidation, pH 6.2 for reduction) containing 20 mM $MgCl_2$ and 8 mM NADP(H). The reaction was started by addition of 10 µL of **2**, **3** or **5** (200 mM in DMSO) to give final concentrations of 4 mM substrate and 2% v/v DMSO. The reaction proceeded at 30 °C in an Eppendorf Thermomixer at 1000 rpm for 16-18 h and was then stopped by addition of 500 µL of acetonitrile. After centrifugation, the supernatant was analyzed by HPLC using a Chromolith Performance RP-18 column with 1.2 mL $min^{-1}$ 1% formic acid and acetonitrile as the mobile phase. The respective HPLC traces are schown in the Supplementary data.
15. Bioconversions with purified enzymes contained 2.7 mg of purified *Yl*SDR at pH 10.5. All other ingredients, workup and analysis see Ref. 14.

## 2.1 Supplementary information

### 2.1.1 Enzyme identification, cloning and expression

The gene sequence of SalM from the marine organism *Salinispora tropica* (GenBank: EF397502.1) was used as a template for a blastx[2] search against the sequenced genome of *Yarrowia lipolytica* (taxid: 4952). The top hits with Accession Nr. XP503594.1 and XP500963.1, respectively, showed alignment scores 80-200, all other hits had scores below 80. XP503594.1 is 33% identical to SalM with 84% sequence coverage and XP500963.1 30% identical with 87% sequence coverage. The protein sequence alignment is depicted in Figure S1.

Both genes were amplified from genomic DNA of the *Yarrowia lipolytica* CLIB122 strain and cloned into the parent vector of pK470, namely pMS470. The latter codes for ampicillin resistance and the proteins of interest are expressed without C- or N-terminal tags in their native forms. After sequence verification, the constructs were transformed into *E. coli* BL21 (DE3) Gold and subjected to expression trials at different temperatures and IPTG concentrations. Whereas XP500963.1 (*Yl*SDR) expressed well under several conditions, no expression of soluble XP503594.1 was observed under any of the tested condition. Consequently, XP500963.1 (*Yl*SDR) was chosen for recloning and further characterization.

```
XP_503594.1          MSNSAKAAVVPPAPTAEDIARANAGSKEEPVFQAKNFLSKFRLDGKVAIVTGGARGLGFS 60
XP_500963.1          MPAPATYATG-LTPLPTPVPKV-----------SKNIMERFSLKGKVASITGSSSGIGFA 48
SalM_ABP73642.1      -----------------------------------MTNGGRLSGKVSLITGAAHGIGHA 24
                                                        : .   *.***: :**.: *:*.:

XP_503594.1          MAEGLCSVGLKGIAILDVQQDLGLDAIEKLHKAYGVQAQFYKADVRDEESVNEIIDRVVH 120
XP_500963.1          VAEAFAQAGAD-VAIWYNSKPS-DEKAEYLSKTYGVRSKAYKCAVTNAKQVETTIQTIEK 106
SalM_ABP73642.1      TAVWFAREGAR--LVVSDVDGAALEKCHAELAESGAEVTTVIADVSDATQAHRMVQTAVD 82
                      *  :.  *     :    .    :  .      *..     . * :  ...  ::   .

XP_503594.1          DLGSVDVVVNSAGVADLVH-AAEYP-ADKFRRVIDINLNGSFLVTQAAARHMIKQGTGGT 178
XP_500963.1          DFGKIDIFIANAGIPWTAGPMIDVPNNEEWDKVVDLDLNGAYYCAKYAGQIFKKQGYG-S 165
SalM_ABP73642.1      VYNRLDVVVANAGVIPLHE--ITEATEQDWDEVMAVDGKGMFLTCKYGIDAMLATGGS-- 138
                       . :*:.: .**:          .  :.: .*: :: :* :   : .   :   * .

XP_503594.1          VVFIASMSGSIVNWPQPQSAYNASKAAVKHLSKSLAAEWAVHNIRCNSISPGYMDTALNR 238
XP_500963.1          FIFTASMSGHIVNIPQMQACYNAAKCAVLHLSRSLAVEWAGF-ARCNTVSPGYMATEISD 224
SalM_ABP73642.1      IVCLSSISG--VAGQRGQSTYGPAKFVASGLTKHLAVECARHCIRVNAVAPGTIRTNRVR 196
                     .:  :*:**  *   : *: *..:* ..  *:: **.* * .  * *:::** : *

XP_503594.1          AYN------TLFEEWKDRTPLGRLGDPDELTGACIYLASDASSYVTGSDIIIDGGYTII 291
XP_500963.1          FIPR-----DTKEKWWQLIPMGREGDPSELAGAYIYLASDASTYTTGADILVDGGYCCP 278
SalM_ABP73642.1      RLEEEPGGPEYLEDIVRLHPAGRLGEPSEVAAAIGFLASDEASFITGVVLPVDGGYLAQ 255
                               *.     * ** *:*.*::.*  :**** ::: **  : :****
```

**Figure S1. Multiple sequence alignment (CLUSTAL 2.1)**

[2] Basic Local Alignment Search Tool of NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi

### 2.1.2 Kinetic characterization of YlSDR

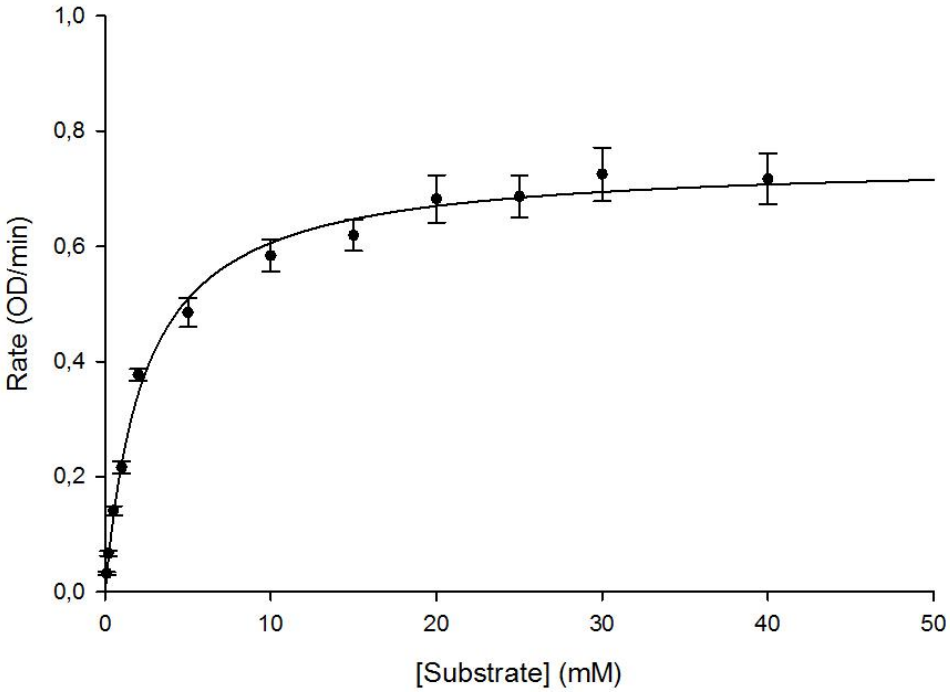


**Figure S2. Saturation curve of the oxidation of rac-2-heptanol;** $K_m$ = 2.36 ± 0.24 [mM], maximal reaction velocity = 0.75 ± 0.02 [U/min], $v_{max}$ = 0.27 ± 0.01 [mM/min], $v_{max,\ spec}$ = 2.13 ± 0.04 [µmol/min mg]

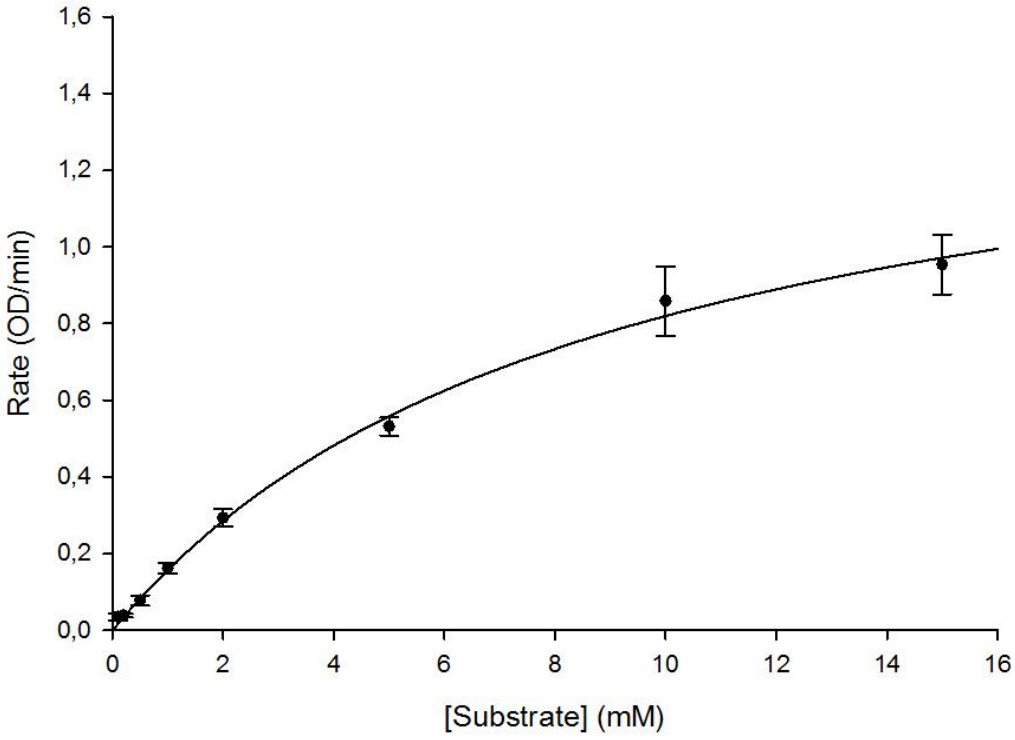


**Figure S3. Saturation curve of the reduction of ribulose;** $K_m$ = 8.80 ± 2.10 [mM], maximal reaction velocity = 1.54 ± 0.18 [U/min], $v_{max}$ = 0.55 ± 0.06 [mM/min], $v_{max,\ spec}$ = 10.19 ± 0.14 [µmol/min mg]

### 2.1.3 Mass spectra and HPLC profiles of authentic standards:

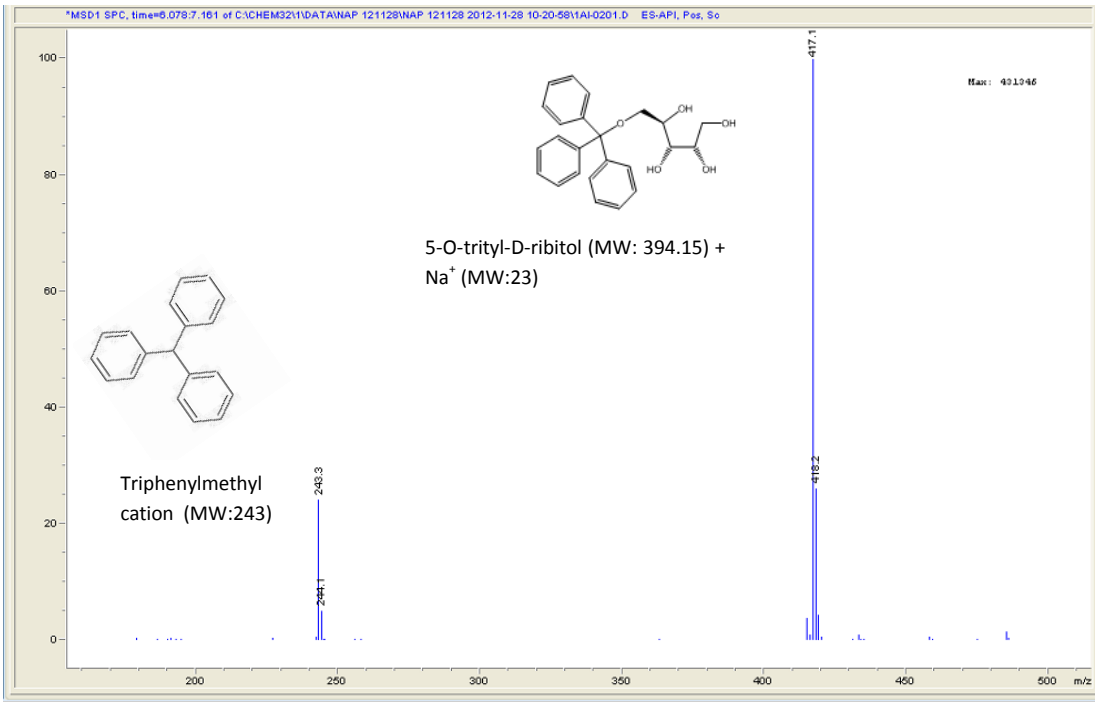


**Figure S4. Mass spectrum of 5-*O*-trityl-ribitol**

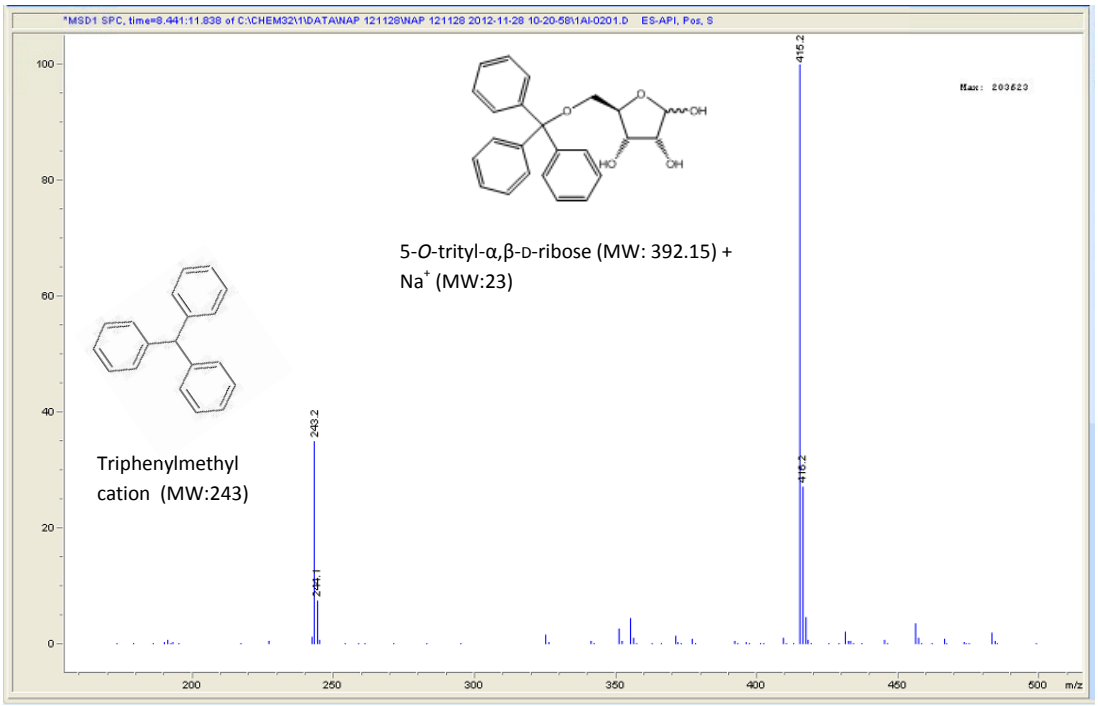


**Figure S5. Mass spectrum of 5-*O*-trityl-α,β-D-ribose**

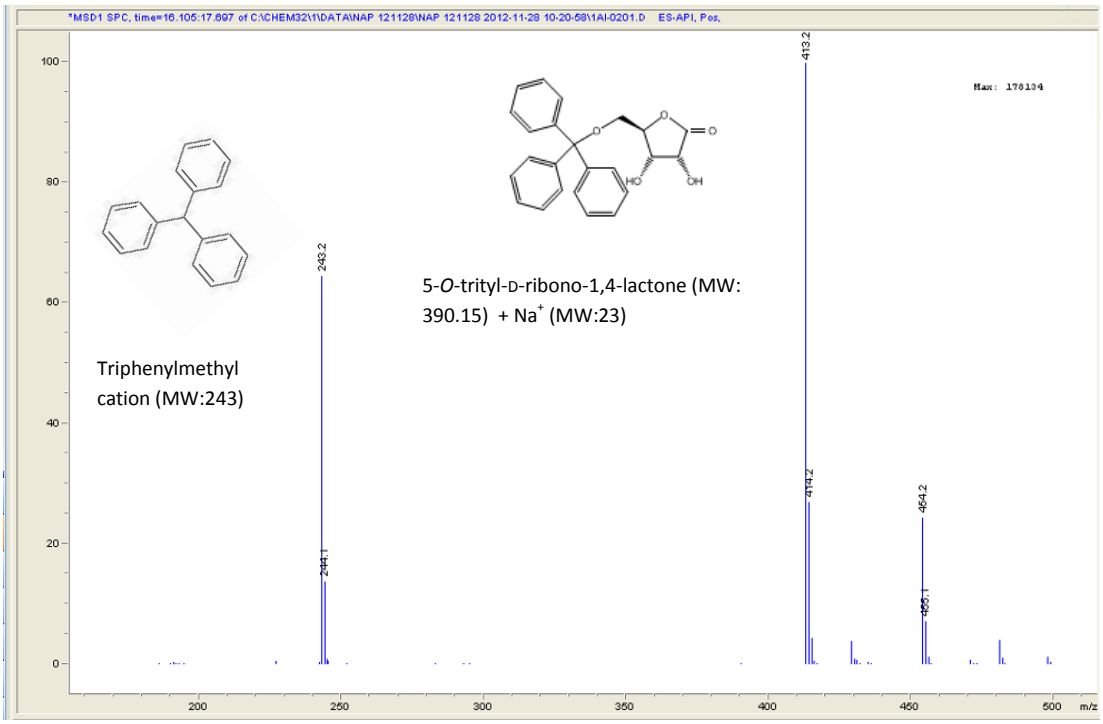


**Figure S6. Mass spectrum of 5-*O*-trityl-D-ribono-1,4-lactone**

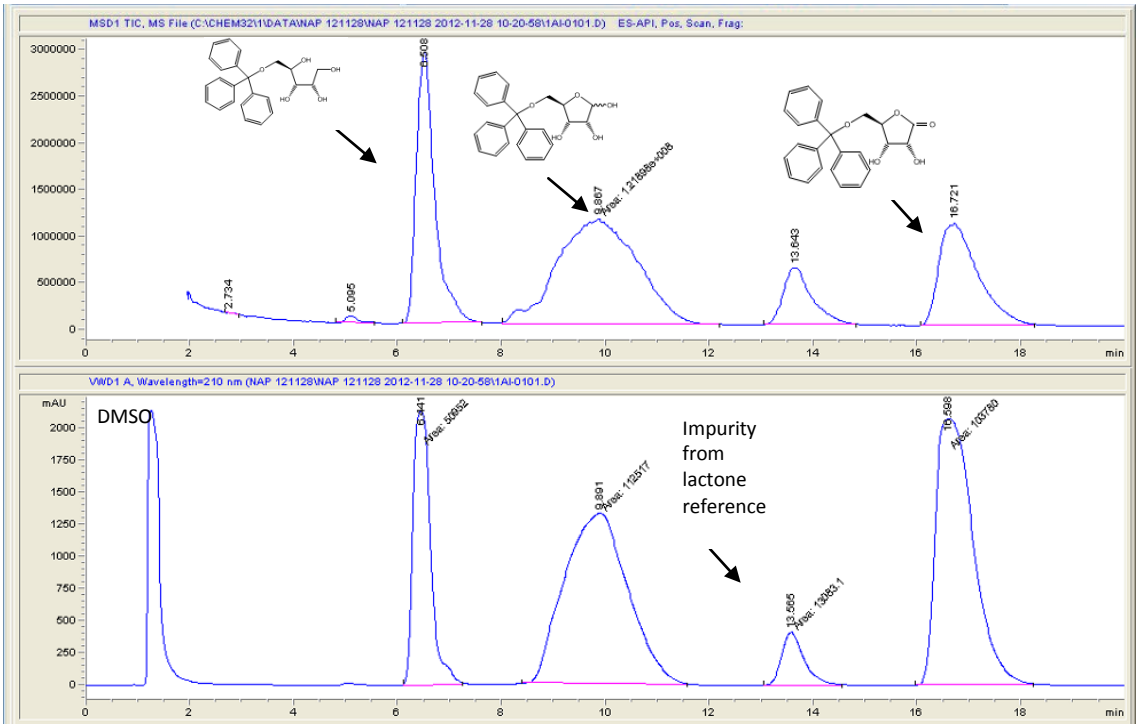


**Figure S7. Positive MS-Scan and HPLC trace at 210 nm of a mixture of authentic standards of 5-*O*-trityl-ribitol, 5-*O*-trityl-α,β-D-ribose and 5-*O*-trityl-D-ribono-1,4-lactone in DMSO**

### 2.1.4 HPLC profiles of bioconversion samples

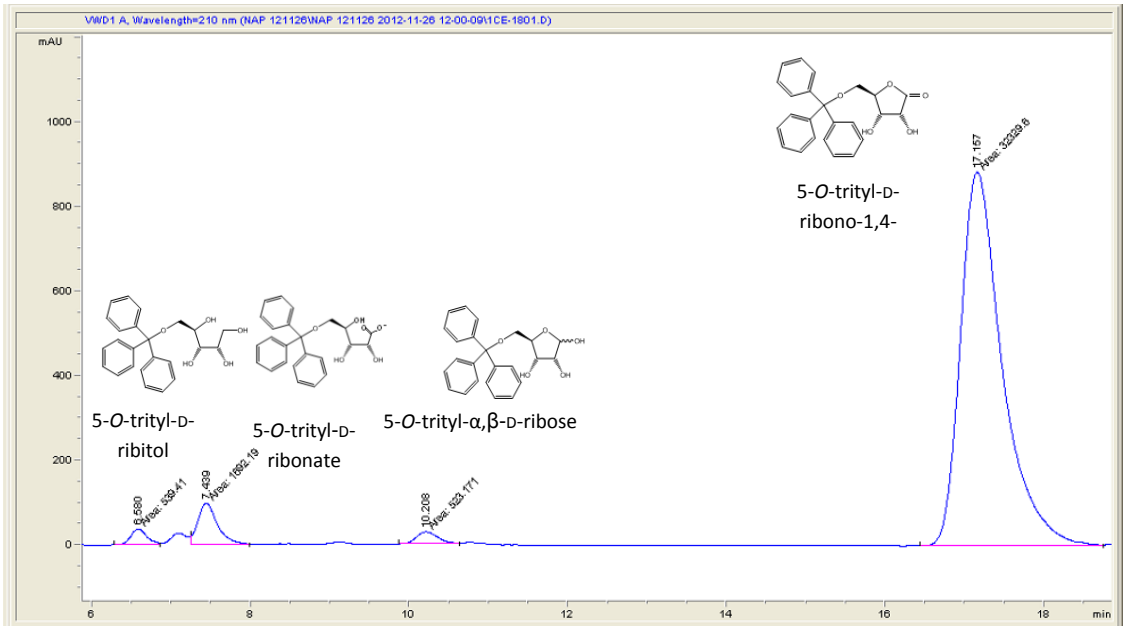


**Figure S8. Oxidation of 5-*O*-trityl-α,β-D-ribose by whole cells of *E. coli* BL21 (DE3) expressing pK470:*Yl*SDR**

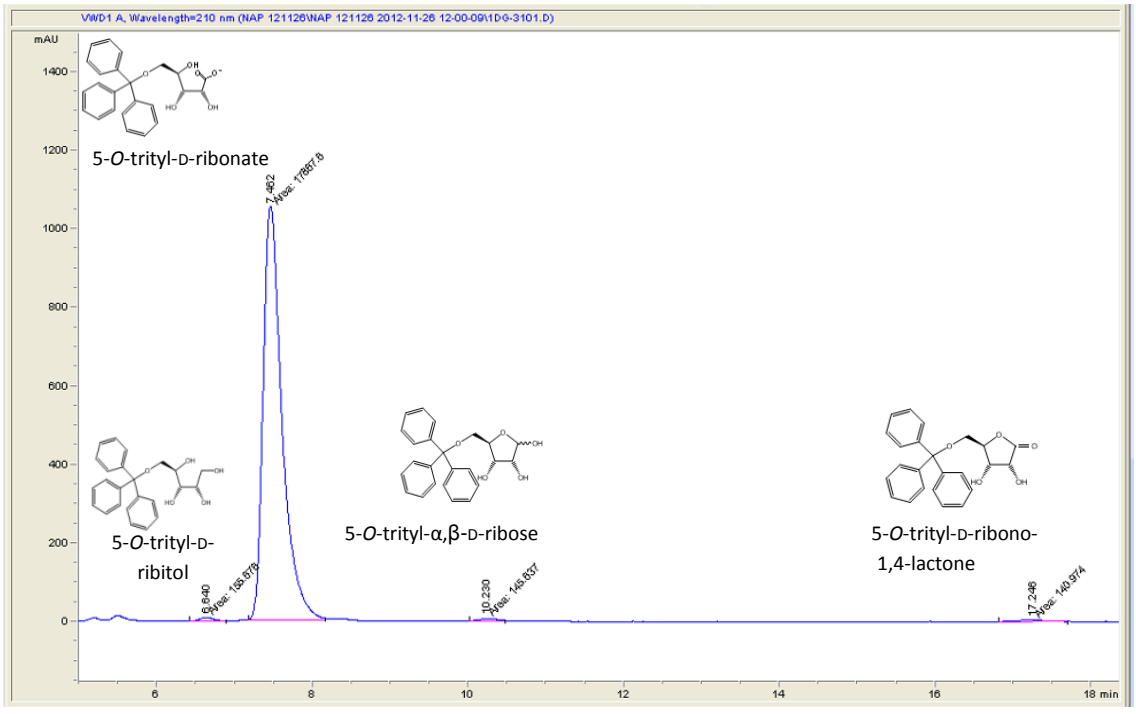


**Figure S9. Oxidation of 5-*O*-trityl-α,β-D-ribose by purified *Yl*SDR**

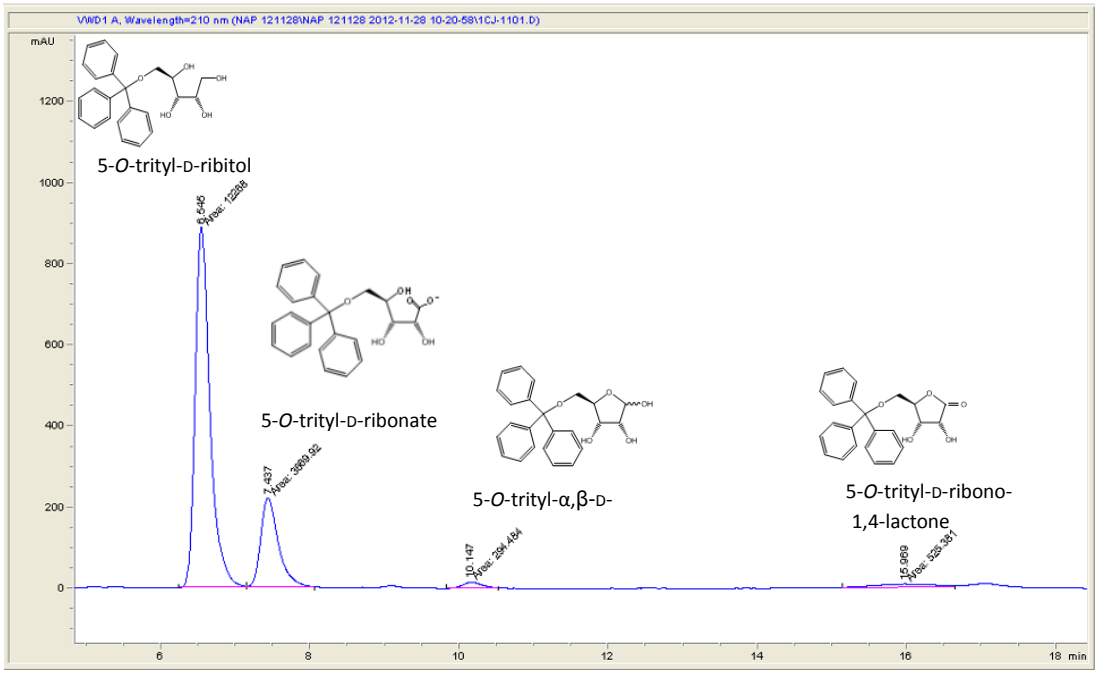


**Figure S10. Oxidation of 5-*O*-trityl-D-ribitol by whole cells of *E. coli* BL21 (DE3) expressing pK470:*Yl*SDR**

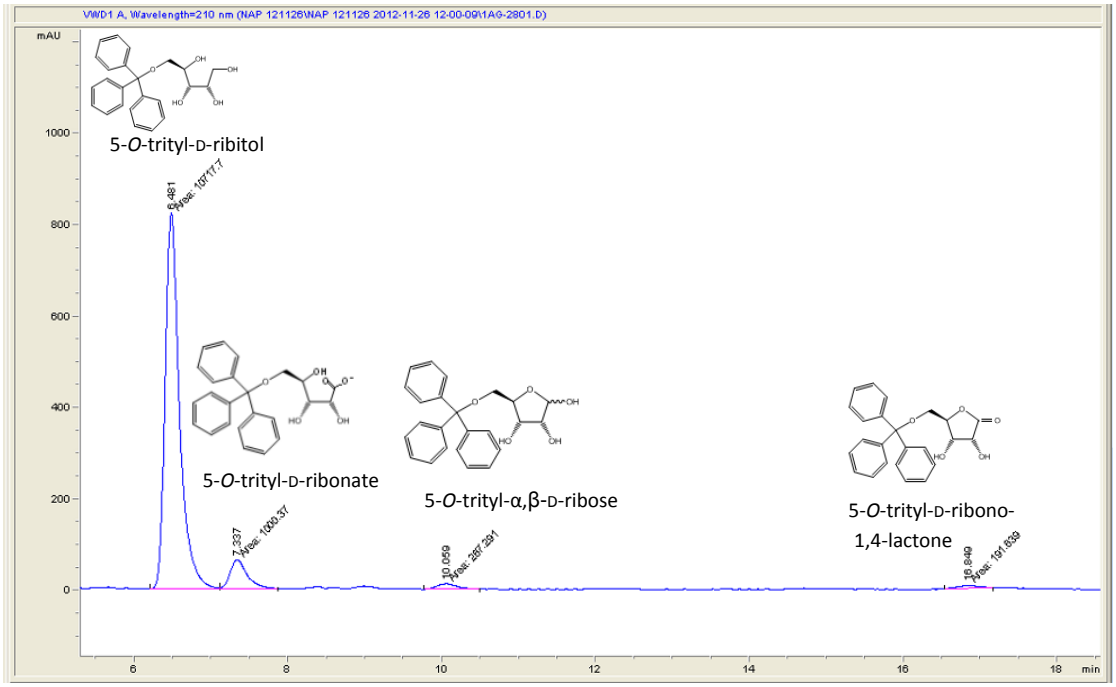


**Figure S11. Oxidation of 5-*O*-trityl-D-ribitol by purified *Yl*SDR**

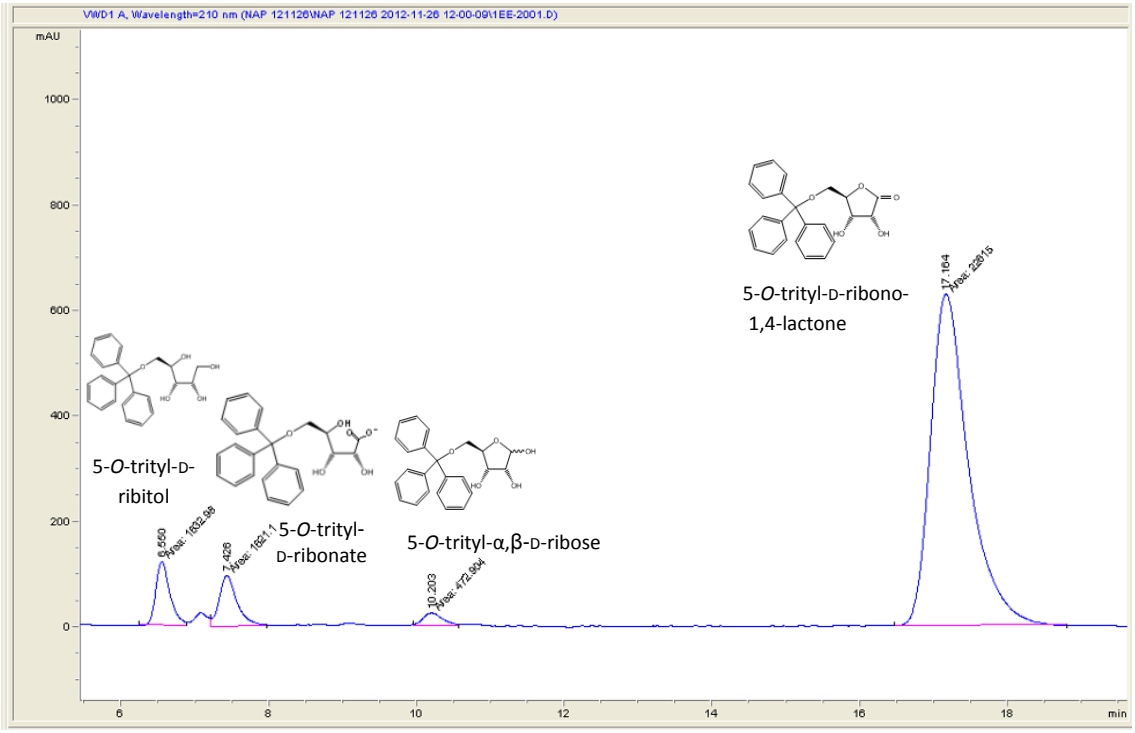


**Figure S12. Reduction of 5-*O*-trityl-α,β-D-ribose by whole cells of *E. coli* BL21 (DE3) expressing pK470:*Yl*SDR**

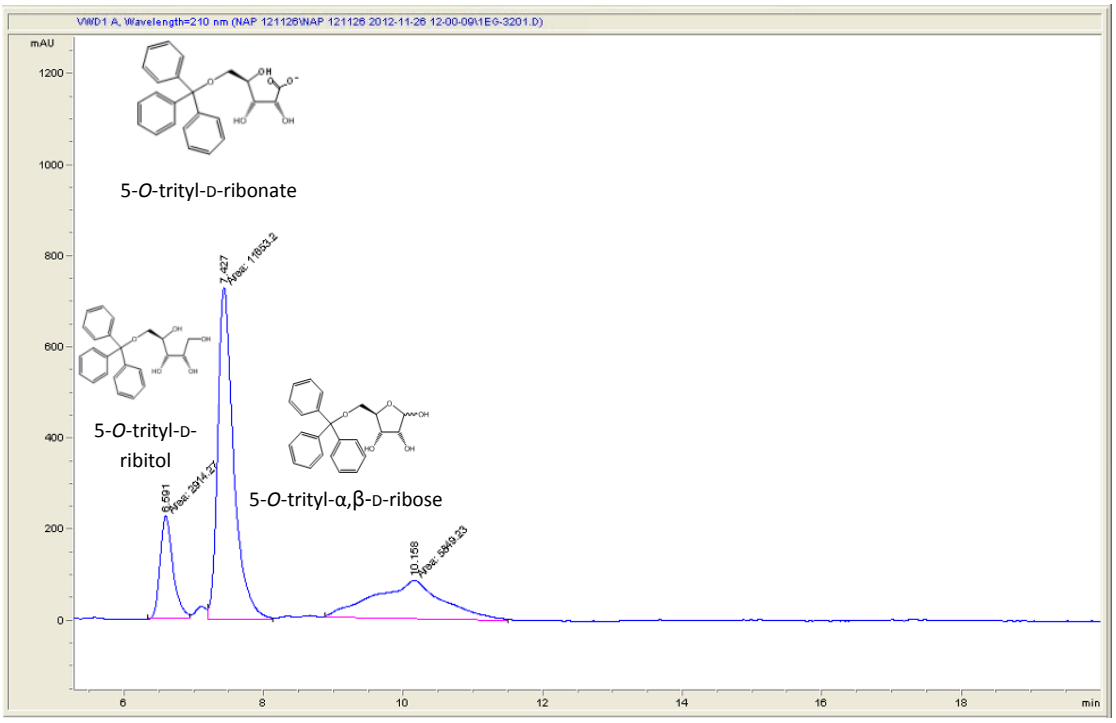


**Figure S13. Reduction of 5-*O*-trityl-α,β-D-ribose by purified *Yl*SDR**

# 3 Enantiocomplementary *Yarrowia lipolytica* oxidoreductases

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# Enantiocomplementary *Yarrowia lipolytica* Oxidoreductases: Alcohol Dehydrogenase 2 and Short Chain Dehydrogenase/Reductase

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**Abstract:** Enzymes of the non-conventional yeast *Yarrowia lipolytica* seem to be tailor-made for the conversion of lipophilic substrates. Herein, we cloned and overexpressed the Zn-dependent alcohol dehydrogenase ADH2 from *Yarrowia lipolytica* in *Escherichia coli*. The purified enzyme was characterized *in vitro*. The substrate scope for *Yl*ADH2 mediated oxidation and reduction was investigated spectrophotometrically and the enzyme showed a broader substrate range than its homolog from *Saccharomyces cerevisiae*. A preference for secondary compared to primary alcohols in oxidation direction was observed for *Yl*ADH2. 2-Octanone was investigated in reduction mode in detail. Remarkably, *Yl*ADH2 displays perfect (*S*)-selectivity and together with a highly (*R*)-selective short chain dehydrogenase/ reductase from *Yarrowia lipolytica* it is possible to access both enantiomers of 2-octanol in >99% ee with *Yarrowia lipolytica* oxidoreductases.

**Keywords:** Zn-dependent alcohol dehydrogenase; *Yarrowia lipolytica*; biooxidation; short chain dehydrogenase/reductase; medium chain secondary alcohols; enantioselective reduction

## 1. Introduction

Chiral alcohols are valuable building blocks for pharmaceuticals and agrochemicals [1] and a multitude of studies have been devoted on biocatalytic methodologies for their production. Nevertheless, there is still a high demand for new enzymes, which operate on specific substrates with high activity and selectivity. Especially, lipophilic compounds are a challenge for classical biocatalysis because substrate availability is low in the aqueous phase in which the enzymes are usually present. The non-conventional yeast *Yarrowia lipolytica* is typically found in lipid-rich media [2] and therefore its enzymes are thought to be evolved to metabolize non-polar substrates [3]. The work of Fantin *et al.* on new alcohol oxidation activities showed, for example, that *Yarrowia lipolytica* alcohol dehydrogenases (ADHs) are highly interesting candidates for biocatalysis [4]. *In vivo*, yeast ADHs are mostly responsible for ethanol formation or consumption and cofactor balance. *In vitro*, ADH1 from *Saccharomyces cerevisiae* (*Sc*ADH1; E.C: 1.1.1.1) is used for cofactor recycling with EtOH as the sacrificial substrate in order to promote NADH dependent enzyme catalyzed reduction [5]. *Sc*ADH1 is a well-studied Zn- and NAD(H) dependent enzyme [6] with known crystal structure (pdb code: 2hcy). The *Yarrowia lipolytica* genome codes for five homologous Zn-dependent ADHs. They are currently filed as putative enzymes [7]. Three of these five proteins were annotated as putative ADH1, ADH2, and ADH3, one as a protein with similarity to putative *Yarrowia lipolytica* ADH3, and one as protein with similarity to mitochondrial ADH3 of *S. cerevisiae*. Of these proteins, ADH2 showed the highest similarity to *Sc*ADH1 and was therefore chosen as a target enzyme (Table 1). *Yl*ADH2 shows sequence similarity to alcohol dehydrogenases from other yeasts [8], e.g., *Pichia stipitis* ADH1 (74% identity) [9], *Candida maltosa* ADH2A [10] (73% identity), *S. cerevisiae* ADH3 (71% identity), and *Hansenula polymorpha* ADH (75% identity) [11]. Whereas *Sc*ADH3-like enzymes from different yeasts are mitochondrial enzymes [3,12], the primary sequences of *Sc*ADH1 and *Yl*ADH2have no mitochondrial targeting sequence according to the PSORTII algorithm [13]. Herein we report the heterologous expression of *Yarrowia lipolytica* ADH2, the enzyme's substrate scope and its enantioselectivity. Further, *Yl*ADH2 is compared to a *Yarrowia lipolytica* short chain dehydrogenase/ reductase (*Yl*SDR), which is enantiocomplementary and offers the possibility to synthesize the other enantiomer of 2-octanol.

**Table 1.** Protein similarities of *Saccharomyces cerevisiae* ADH1 and *Yarrowia lipolytica* Zn-dependent ADHs. Italics: identities (%), bold: positives (%).

| | ***Sc*ADH1** | ***Yl*ADH1** | ***Yl*ADH2** | ***Yl*ADH3** | ***Yl*ADH** | ***Yl*ADH** |
|---|---|---|---|---|---|---|
| Acc. Nr.: | NP_014555 | XP_503282 | XP_504077 | XP_500127 | XP_500087 | XP_503672 |
| NP_014555 | 100 | *68* | *68* | *69* | *66* | *54* |
| XP_503282 | **80** | 100 | *94* | *98* | *81* | *57* |
| XP_504077 | **82** | **98** | 100 | *94* | *79* | *56* |
| XP_500127 | **80** | **99** | **97** | 100 | *82* | *58* |
| XP_500087 | **80** | **90** | **85** | **90** | 100 | *53* |
| XP_503672 | **70** | **70** | **70** | **71** | **69** | 100 |

## 2. Results and Discussion

In continuation of our search for versatile oxidoreductases especially for lipophilic compounds [14], we amplified the ADH2 gene from genomic DNA of the *Yarrowia lipolytica* CLIB122 strain and cloned it into two different vector systems. In addition to the native ADH2 sequence, an *N*-terminal His-tag was introduced to facilitate enzyme purification. *Yl*ADH2 expression in the pEHisTEV vector [15] – that adds an *N*-terminal His-tag and a TEV protease cleavage site to the protein of interest – with the T7 promoter resulted in approximately identical expression level compared to untagged *Yl*ADH2 expressed from pMS470, a vector with tac promoter [16] (see Figures S1 and S2 in the supplementary information). His-tagged *Yl*ADH2 was then purified by Ni-affinity chromatography and used for *in vitro* characterization. Investigation of the cofactor specificity revealed, as expected, a strong preference of *Yl*ADH2 for NAD(H) over NADP(H) [6]. We were particularly interested in the substrate tolerance of *Yl*ADH2 and investigated the oxidation of the following substrates: EtOH, 2-propanol, 1-butanol, (2*R*,3*R*)-butanediol, cyclohexanol, 4-methyl-2-pentanol, *rac*-2-heptanol, 1-octanol, *rac*-2-octanol, (*R*)-2-octanol, (*S*)-2-octanol, 1-nonanol, *rac*-2-nonanol, 1-decanol, 1-dodecanol, 1-phenylethanol, (*R*)-2-amino-2-phenylethanol, (*S*)-2-amino-2-phenylethanol, phenylacetaldehyde, adonitol, arabitol, xylitol, sorbitol, and mannitol. Due to the lipophilicity of long chain alcohols, surfactants were used to increase their solubility under assay conditions [17].

**Table 2.** Exploration of the substrate spectrum of *Yl*ADH2.

| Entry | Substrate | Relative oxidation activity (%) | Substrate | Relative reduction activity (%) |
|---|---|---|---|---|
| 1 | 2-propanol | 53 | acetone | <5 |
| 2 | 1-butanol [a] | 9 | | |
| 3 | *rac*-4-methyl-2-pentanol | 6 | | |
| 4 | *rac*-2-heptanol [a] | 64 | | |
| 5 | 1-octanol [b] | 7 | | |
| 6 | *rac*-2-octanol [a] | 100 [c] | 2-octanone | 100 [d] |
| 7 | 1-nonanol [b] | 7 | | |
| 8 | *rac*-2-nonanol [a] | 81 | 2-nonanone | 106 |
| 9 | 1-decanol [b] | 6 | | |
| 10 | *rac*-2-decanol [a] | 77 | 2-decanone | 100 |

[a] Tween 20 was used as solubilizer at 0.45% v/v end concentration; [b] Tween 20 was used as solubilizer at 0.75% v/v end concentration. 0.75% Tween 20 reduced the activity towards *rac*-2-octanol oxidation by 20% compared to 0.45%; [c] 100% corresponds to 1.1 ± 0.1 U·mg$^{-1}$; [d] 100% corresponds to 0.50 ± 0.06 U·mg$^{-1}$.

Ethanol is by far the best substrate for ADH1 from *Saccharomyces cerevisiae* [6] and *Yl*ADH2 can also oxidize EtOH, however, its specific activity is two orders of magnitude lower than that of a commercial preparation of *Sc*ADH1 (>300 U/mg as specified by the manufacturer). In this study, the above-mentioned compounds were subjected to both *Yl*ADH2 and *Sc*ADH1 oxidation. Except for EtOH (see above) and 2-propanol (197 ± 77

mU/mg), *Sc*ADH1 showed no significant activity for any substrate. *Yl*ADH2 exhibited a much broader substrate tolerance than its homolog from *Saccharomyces cerevisiae* (Table 1). Substrates which showed less than 5% of the activity towards 2-octanol oxidation are not listed in Table . The highest specific activity was observed for the oxidation of racemic 2-octanol (1.1 ± 0.1 $U \cdot mg^{-1}$), which is a value similar to that observed for *Yarrowia lipolytica* short chain dehydrogenase/reductase *Yl*SDR (NCBI Accession Nr. XP_500963.1) [14]. Both enzymes clearly preferred secondary to primary alcohols. However, in contrast to *Yl*SDR, *Yl*ADH2 was not able to oxidize carbohydrate substrates.

The optimal reaction temperature for the oxidation of racemic-2-octanol was determined between 25 °C and 37 °C. *Yl*ADH2 showed a plateau of highest activity between 28 °C and 33 °C. The optimal pH of the reaction is strongly dependent on the reaction buffer as depicted in Figure 1. Whereas high activities were observed at pH 9.5 in carbonate and glycine buffer, the same pH was detrimental in borate buffer. A similarly negative effect of borate buffer was also observed for the *Yl*SDR enzyme.

**Figure 1.** pH optimum of *Yl*ADH2 catalyzed oxidation of (*S*)-2-octanol. ♦: citrate;
■: potassium phosphate; ▲: Tris-HCl; ▬: borate; X: glycine; ●: carbonate.

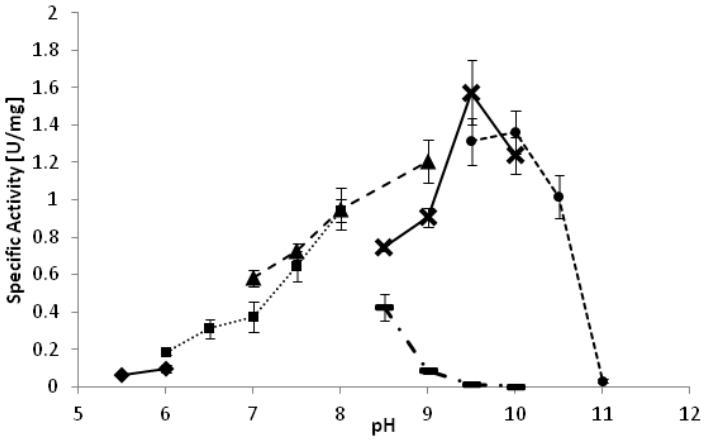
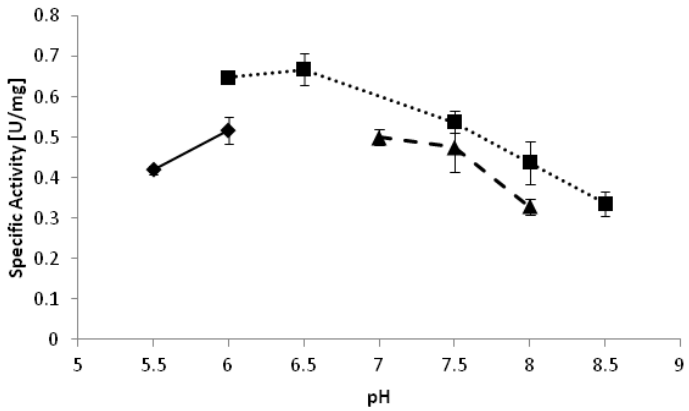



**Figure 2.** pH optimum of *Yl*ADH2 catalyzed reduction of 2-octanone. ♦: citrate; ■: potassium phosphate; ▲: Tris-HCl.



*Yl*SDR was catalyzing the reduction of several substrates and displayed its highest activity for ribulose [14]. *Yl*ADH2, by contrast, was highly specific for medium chain lipophilic ketone substrates among those tested (see experimental section). Relative specific activities for substrate reduction are shown in Table  and the absolute values were approximately 0.5 $U \cdot mg^{-1}$. The optimal pH of the reduction of 2-octanone appeared to be pH 6.5 (Figure 2). Interestingly, reduction reactions often proceed better at relatively low pH as compared to oxidations [18,19]. In the mechanism of a reduction reaction, a hydride is transferred from the nicotinamide donor to the substrate simultaneously to the addition of a proton. At a low pH, the amino acid residues of the protein are predominantly protonated, which facilitates the proton transfer. In oxidation direction, a proton needs to be removed from the substrate, typically from a basic amino acid residue in the active site. In this case, the deprotonated state of the protein at elevated pH seems to be beneficial.

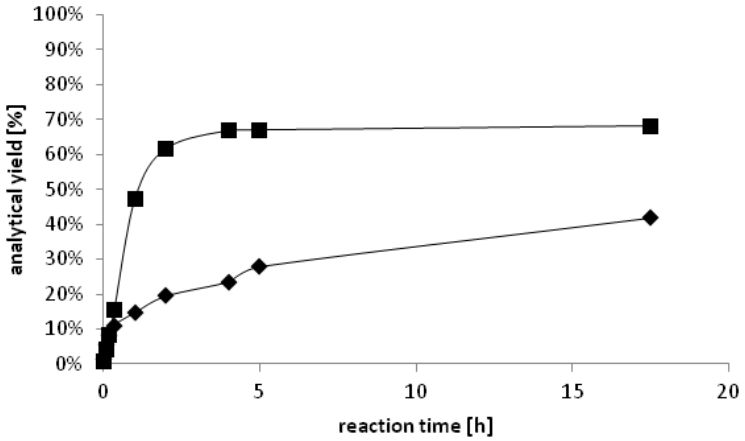
The determination of kinetic parameters for *Yl*ADH2 mediated oxidation and reduction showed that the $k_{cat}$ value for reduction of 2-octanone is approximately half of the value of the respective oxidation (Table 3).

**Table 3.** Apparent kinetic parameters for *Yl*ADH2.

| | **Oxidation** | | **Reduction** |
|---|---|---|---|
| | (*S*)-2-octanol | $NAD^+$ | 2-octanone |
| $K_m$ [mM] | 1.42 ± 0.03 | 17.8 ± 1.26 | 5.38 ± 0.76 |
| $k_{cat}$ [$s^{-1}$] | 1.05 ± 0.52 | 3.43 ± 0.05 | 0.56 ± 0.04 |
| $k_{cat}/K_m$ [$s^{-1} \cdot mM^{-1}$] | 0.74 ± 0.50 | 0.19 ± 0.07 | 0.10 ± 0.16 |

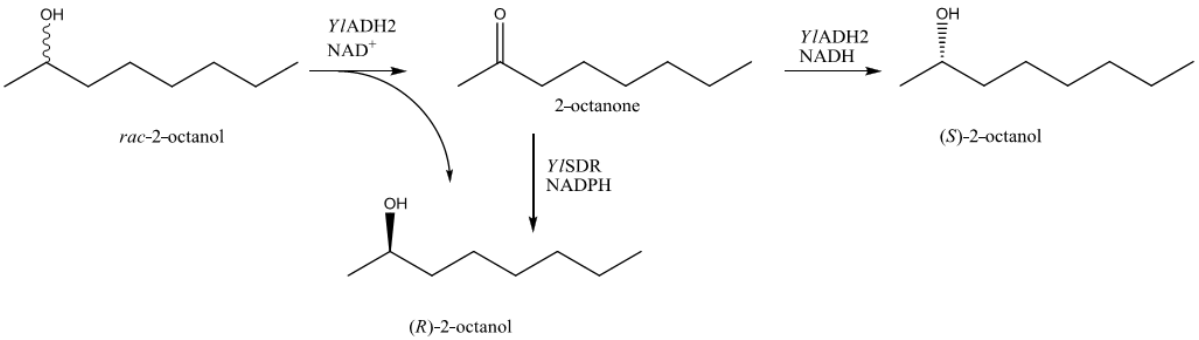
Alcohol dehydrogenases are often used as catalysts for enantioselective syntheses – on laboratory scale and in industrial processes [20,21]. To determine the enantiopreference of *Yl*ADH2, the single enantiomers of 2-octanol were subjected to $NAD^+$ mediated oxidation. The monitored NADH formation was significantly faster in case of (*S*)-2-octanol (1.1 $U \cdot mg^{-1}$) compared to the (*R*)-enantiomer (<0.2 $U \cdot mg^{-1}$) – a first indication for (*S*)-selectivity of the enzyme. In order to verify this result, we investigated the reaction in reduction direction. Therefore, 2-octanone was used as the substrate and the products were analyzed by chiral gas chromatography after derivatization to the corresponding acetates. *Yl*ADH2 produced exclusively the (*S*)-enantiomer (>99% *ee*). By contrast, the (*R*)-enantiomer was obtained in >99% *ee* in case that *Yl*SDR was applied as the biocatalyst (Figure 3).

**Figure 3.** Time dependent formation of ♦ (*S*)-2-octanol (<99% *ee*) catalyzed by *Yl*ADH2 and ■ (*R*)-2-octanol (<99% *ee*) catalyzed by *Yl*SDR.




In order to improve the conversions, we applied cofactor recycling, using glucose dehydrogenase (GDH) and formate dehydrogenase (FDH) [22] in different combinations of enzyme and co-substrate concentrations. The cofactor recycling system GDH/glucose gave moderate conversions in comparison to FDH/formate. Using 0.02 U of FDH in combination with sodium formate (100 mM) at 0.5 mL scale, 70% of 2-octanone were reduced to (*S*)-2-octanol in >99% *ee* within one hour. After 2.5 h, the conversion was 83% and full conversion (>99% *ee*) was observed after a reaction time of <16 h. Enantiomerically pure lipophilic alcohols can be used as derivatizing agent for the enantioseparation of carboxylic acids [23] or e.g., for the preparation of functional materials. (*S*)-2-Octanol, for instance, was used as the chiral selector in microemulsion electrokinetic chromatography [24]. The (*R*)-enantiomer served as a precursor for chiral liquid crystals [25]. The two *Yarrowia lipolytica* oxidoreductases described herein offer the possibility to produce both enantiomers of 2-octanol in highly pure form, possibly by oxidative kinetic resolution of racemic 2-octanol, or by the reduction of prochiral 2-octanone (Scheme 1).

**Scheme 1.** Routes to enantiomerically pure (*S*)- and (*R*)-2-octanol via *Yarrowia lipolytica* oxidoreductases.



## 3. Experimental

### *3.1. General*

*Yarrowia lipolytica* CLIB 122 (supplementary information Figure S3) was obtained from Centre International de Ressources Microbiennes (CIRM, France). *E. coli* cells were cultivated in RS 306 and Multitron shakers (Infors AG), and the cells were harvested with Avanti centrifuge J-20 (Beckman Coulter). Cell pellets were disrupted with a Branson 102C converter, power was supplied with a Branson Sonifier 250 or a French Press model and cell free extract was obtained by centrifugation in Ultracentrifuge Optima LE80K (Beckman Coulter). Enzymes were purified using a HisTrap$^{TM}$ FF 5 mL column on an ÄKTA Purifier 100 with Frac-950, software Unicorn 4.11, and desalted using a HiPrep$^{TM}$ 26/10 Desalting column on an ÄKTA Prime, software PrimeView 5.0 (GE Healthcare Life Sciences). Protein samples were analyzed with 4–12% NuPAGE® Bis-Tris Gel (Invitrogen) and photometric measurements were carried out on Synergy Mx plate reader (BioTek) using the Gen5.11 Software. Chiral GC analyses were carried out on a Hewlett-Packard 6890 instrument. NADH and $NAD^+$ (sodium salt; 97% pure) was obtained from Roche Diagnostics. GDH was obtained from DSM Innovative Synthesis BV. 2-Nonanone and 2-decanone were purchased from Alfa Aesar and all other chemicals including alcohol dehydrogenase from *Saccharomyces cerevisiae* (lyophilized powder, ≥300 $U \cdot mg^{-1}$, order number A7011) were purchased from Sigma–Aldrich/Fluka and used as received.

### *3.2. Isolation of Genomic DNA and Gene Cloning*

Genomic DNA from *Yarrowia lipolytica* strain CLIB 122 was isolated according to the published procedure [26].

The fragment corresponding to *Yl*ADH2 was amplified from genomic DNA using Phusion® High-Fidelity DNA polymerase (Finnzymes) with the following primers: pEHisTEVmutYlADH2_f: 5'-TAC GAG ATA TCA TGT CTG CTC CCG TCA TCC CC-3'; pEHisTEVmutYlADH2_r: 5'-TAA CTG CGG CCG CTT ACT TGG AGG TGT-3'. The *Eco*RV and *Not*I restriction sites are underlined. The gene was cloned into the pEHisTEV vector, previously digested with *Eco*RV and *Not*I.

*N*-Terminally tagged and untagged *Yl*ADH2 were cloned into vector pMS470 as follows: the fragments were amplified from pEHisTEV:ADH2 using Phusion® High-Fidelity DNA polymerase (Finnzymes) and following primers: pMS470d8ADH2_f 5'-TAT CAC ATA TGT CTG CTC CCG TCA TC-3'; pMS470d8ADH2_r 5'-TTT CTG CAT GCT TAC TTG GAG GTG TC-3'; pMS470d8_HIS-TEVADH2_f 5'-ATA CAT ATG TCG TAC TAC CAT CAC CAT CAC C-3' and pMS470d8_HIS-TEVADH2_r 5'-ATA GCA TGC TTA CTT GGA GGT GTC CAG-3'; The restriction sites *Nde*I and *Sph*I are underlined. Amplification conditions were: 98 °C for 5 min, followed by 30 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, then a final incubation of 72 °C for 7 min. The PCR products were gel separated and the excised DNA was purified with the QIAquick Gel Extraction Kit. The DNA was digested

with *Nde*I and *Sph*I restriction enzymes (Fermentas) in the presence of Tango buffer (Fermentas) and column purified according to QIAquick PCR purification protocol. The genes were cloned into the pMS470 vector, previously digested with *Nde*I and *Sph*I and dephosphorylated with Calf Intestine Alkaline Phosphatase (Fermentas) in the presence of FastDigest buffer (Fermentas), using T4 polymerase (Fermentas) in T4 DNA ligase buffer (Fermentas), at room temperature for 1 h.

The fragment corresponding to *Yl*SDR was amplified using Phusion® High-Fidelity DNA polymerase (Finnzymes) with the following primers: YaliSDR2470_f: 5'-AAT CAC ATA TGC CTG CAC CAG CAA CCT AC-3' and YaliSDR2470_r: 5'-AAT CAG CAT GCT CAA GGA CAA CAG TAG CC-3'. The *Nde*I and *Sph*I restriction sites are underlined. Amplification conditions were: 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s, then a final incubation of 72 °C for 7 min. The PCR products were gel separated and the excised DNA was purified with the QIAquick Gel Extraction Kit (QIAGEN). The DNA was digested with *Nde*I and *Sph*I restriction enzymes (Fermentas) in the presence of Tango buffer (Fermentas) and column purified according to the QIAquick PCR purification protocol. The gene was cloned into the pK470 vector, which contained an *N*-terminal His-Tag (for the vector map, see Figure S4 supplementary information). The pK470 vector was digested with *Nde*I and *Sph*I and gel purified according the procedure described above, prior to the ligation. Ligation was carried out with T4 polymerase (Fermentas) in T4 DNA ligase buffer (Fermentas), at room temperature for 1 h.

The constructs were transformed into electrocompetent *E. coli* TOP10 F' cells (Invitrogen) and cells were plated out on LB with 50 μg/mL kanamycin (for pEHisTEV and pK470) or 100 μg/mL ampicillin (for pMS470). The plasmids were isolated with the GeneJET™ Plasmid Miniprep Kit (Fermentas) and the sequences confirmed by LGC genomics. The plasmids were then transformed into electrocompetent *E. coli* BL21 (DE3) Gold cells (Stratagen).

*3.3. Expression and Purification*

Expression and purification of *Yl*SDR was carried out as described previously (supplementary information Figure S5) [14]. *E. coli* BL21 (DE3) Gold harboring ADH2 plasmids were cultivated as follows: overnight cultures [50 mL LB with 50 μg/mL kanamycin (for pEHisTEV) or 100 μg/mL ampicillin (for pMS470)] were inoculated with a single colony and grown overnight at 37 °C in an orbital shaker at 110 rpm. 500 mL LB medium with the appropriate antibiotic in 2-L baffled Erlenmeyer flasks were inoculated to an OD of 0.1. These main cultures were grown at 37 °C and 110 rpm to an OD of 0.4–0.6, cooled on ice for 30 min, induced with 0.5 mM of IPTG and supplemented with 0.25 mM $ZnSO_4$ [27]. The cultures were incubated for 20 h at 16 °C and 23 × *g*. The cells were harvested by centrifugation (2,831 × *g*, 4 °C, 10 min), washed with buffer, and disrupted by sonication or French press treatment in Tris-HCl buffer (40 mM; 0.3 M NaCl, pH 8.5). After centrifugation at 72,647 × *g*, 4 °C for 1 h, the cell free extract was either stored at −20 °C or subjected to Ni-

affinity chromatography, re-buffered into potassium phosphate buffer (50 mM, 500 mM NaCl, 40 mM KCl pH 8.5), concentrated with Vivaspin 20 (Sartorius Stedim Biotech S.A), shock frozen in liquid nitrogen, and stored at −80 °C. Protein concentrations were determined using the Bradford method.

*3.4. Substrate Scope*

Alcohol dehydrogenase activity of recombinant *Yl*ADH2 and commercial *Sc*ADH1 were determined by following the reduction of $NAD(P)^+$ at 340 nm in UV-Star Polystyrene plates (Greiner Bio-One). Specifically, 20 µL substrate solution (various alcohols and sugars, 100 mM in 50 mM potassium phosphate, 40 mM KCl, pH 8.5) was added to 140 µL potassium phosphate (50 mM, 40 mM KCl, pH 8.5), followed by 20 µL enzyme solution (0.05–0.1 mg/mL; *Sc*ADH1 dissolved freshly in 10 mM sodium phosphate, pH 7.5; purified *Yl*ADH2 was thawed on ice and diluted appropriately). The reaction was started by addition of 20 µL $NAD^+$ (or $NADP^+$; 10 mM in water) and monitored at 28 °C for *Yl*ADH2 and 30 °C for *Sc*ADH1 for 10 min. The following substrates were investigated: EtOH, 2-propanol, 1-butanol, (2*R*,3*R*)-butanediol, cyclohexanol, 4-methyl-2-pentanol, *rac*-2-heptanol, 1-octanol, *rac*-2-octanol, (*R*)-2-octanol, (*S*)-2-octanol, 1-nonanol, *rac*-2-nonanol, 1-decanol, 1-dodecanol, 1-phenylethanol, (*R*)-2-amino-2-phenylethanol, (*S*)-2-amino-2-phenylethanol, phenylacetaldehyde, adonitol, arabitol, xylitol, sorbitol, and mannitol. To substrates with limited water solubility, 4.5% or 7.5% v/v of Tween 20 was added to the 100 mM substrate stock. In case of phenylacetaldehyde, the addition of 50% DMSO was necessary to ensure a homogenous reaction mixture. Each reaction was performed at least in two sets of quadruple measurements. Blanks without substrate were subtracted. Activity units are defined as the amount of enzyme producing 1 µmol of NADH per min. Specific activity was expressed as units per mg of protein.

The reduction of acetone, cyclohexanone, octanal, 2-octanone, 2-nonanone, 2-decanone, 2-dodecanone, acetophenone, phenylacetaldehyde, ribose, arabinose, xylose, glucose, mannose, lactose, and fructose was monitored at 340 nm *via* the oxidation of NADH in UV-Star Polystyrene plates (Greiner Bio-One). The conditions above were used with the following modifications: 4.5% v/v of Tween 20 was added to the 100 mM substrate stock solution of 2-ketones and 10% Triton to octanal. The reaction was carried out at pH 7.0 and 28 °C and it was started by addition of 20 µL NADH (7.5 mM in water). Activity units are defined as the amount of enzyme consuming 1 µmol of NADH per min.

*3.5. Determination of pH Optima*

Optimal oxidation pH was determined by following the reduction of $NAD^+$ as described in section "substrate scope". (*S*)-2-Octanol was used as the substrate. For the different pH points, the following buffers were used, each in 50 mM concentration containing 40 mM KCl: citrate (pH 5.5–6.0), potassium phosphate buffer (pH 6.0–8.0), TrisHCl (pH 7.0–9.0), borate (pH 8.5–10.0), glycine (pH 8.5–10.0), and carbonate buffer (pH 9.5–11.0). Similarly, the optimal

pH for reduction was determined using 2-octanone as the substrate with above mentioned buffers.

*3.6. Determination of Kinetic Parameters*

The kinetic parameters for (*S*)-2-octanol oxidation and 2-octanone reduction as well as $NAD^+$ reduction were determined. (*S*)-2-octanol was used in concentrations from 0.5 mM to 15 mM and assayed in potassium phosphate buffer (50 mM, 40 mM KCl, pH 8.5). 2-Octanone was used in concentrations from 1 mM to 40 mM and assayed in potassium phosphate buffer (50 mM, 40 mM KCl, pH 7.0). The stock solutions contained 4.5% Tween 20 or less. Kinetic parameters for $NAD^+$ were determined by oxidation of (*S*)-2-octanol (10 mM) in potassium phosphate buffer (50 mM, 40 mM KCl, pH 8.5) with concentrations of $NAD^+$ from 200 μM to 50 mM. All assays were performed as described in section "substrate scope" at 28 °C. The results were evaluated based on Michaelis - Menten kinetics, using SigmaPlot™ version 11.0 for non-linear fitting.

*3.7. Determination of Enantioselectivity*

**(*S*)-2-octanol** was prepared under the following conditions: purified *Yl*ADH2 in potassium phosphate buffer (50 mM, containing 40 mM KCl, pH 6.5) was mixed with 2-octanone (100 mM in the same buffer with 7.5% v/v Tween 20) and NADH (100 mM in water) to give 0.16 mg/mL, 10 mM and 11 mM end concentration, respectively, in total volumes of 500 μL. The reaction proceeded at 28 °C in an Eppendorf Thermomixer at 600 rpm. For each time-point, an extra sample was sacrificed. Substrate and products were extracted into 500 μL of ethyl acetate. A triethylamine-4-(dimethylamino)-pyridine stock solution [TEA-DMAP stock solution: DMAP (8.9 mg, 73.2 μmol) dissolved in TEA (2.00 mL, 14.3 mmL)] was added to the sodium sulfate-dried ethyl acetate extract of the reaction (500 μL, contains ≤ 10.0 mM of 2-octanol) (TEA: 68.3 μL, 490 μmol, 98 equivalents.; DMAP: 0.31 mg, 2.5 μmol, 0.5 equivalents) and acetic anhydride (23.6 μL, 250 μmol, 50 equivalents). After keeping the mixture at 40 °C for 3 h, the reaction was quenched by adding saturated sodium chloride solution (300 μL) and subsequent vigorous shaking. Finally, the ethyl acetate layer was directly subjected to GC analysis on a Chirasil-Dex CB column (25 m × 0.32 mm; 0.25 μm film; Varian). The GC settings were as follows: injector 220 °C; 1.0 bar constant pressure $H_2$ flow; temperature program: initial temperature 60 °C, 85 °C/rate 1.5 °C per min, hold 3 min; The absolute configuration of 2-octanol was assigned by comparison of the elution order on chiral GC with literature known data [28] and by derivatization of commercial (*R*)- and (*S*)-2-octanol. Retention times were 8.0 min for 2-octanone, 13.9 min for (*S*)-octan-2-yl acetate and 17.2 min for (*R*)-octan-2-yl acetate.

For cofactor recycling, the same conditions as describe above were used, with the exception that also 0.03 U of FDH and 100 mM of sodium formate were added to the reaction and the content of NADH cofactor was reduced to 1 mM.

**(*R*)-2-octanol** was prepared under the following conditions: purified *Yl*SDR in citrate buffer (50 mM, pH 5.0) was mixed with 2-octanone (100 mM in the same buffer with 3% v/v Tween 20) and NADPH (100 mM in water) to give 0.11 mg/mL, 10 mM and 11 mM end concentration, respectively, in total volumes of 500 µL. The reaction proceeded at 28 °C in an Eppendorf Thermomixer at 600 rpm. Workup and analysis were carried out as described for (*S*)-2-octanol.

## 4. Conclusions

In conclusion, we have shown that *Yarrowia lipolytica* harbors versatile oxidoreductases that catalyze selective oxidation and reduction reactions. From the two enzymes described herein, secondary alcohols are the preferred substrates in the oxidation direction compared to primary alcohols and aldehydes. Medium chain length ketones with the carbonyl function at position C-2 are reduced to the corresponding secondary alcohols in enantio-complementary form: whereas *Yl*ADH2 produced the (*S*)-enantiomer in >99% *ee*, the (*R*)-enantiomer was obtained with *Yl*SDR.

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## Conflict of Interest

The authors declare no conflict of interest.

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## 3.1 Supplementary information

**Figure S1. *Y. lipolytica* CLIB 122 on YPD medium**

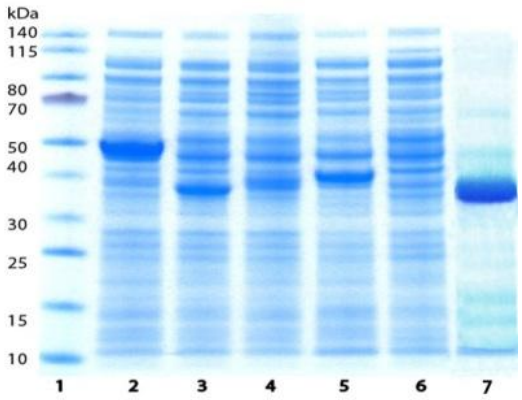



**Figure S2. NuPAGE gel of *Yl*ADH2 and *Yl*HTADH2 cell free extracts and purified *Yl*HTADH2;** Lane 1: PageRuler Prestained Protein Ladder (Fermentas); Lane 2: pMS470:d8 vector control with stuffer fragment; Lane 3: pMS470:*Yl*ADH2, Lane 4: pMS470:*Yl*HTADH2, Lane 5: pEHisTEV:*Yl*ADH2; Lane 6: pEHisTEV vector control; Lane 7: purified *Yl*ADH2; expected size of *Yl*ADH2 is 37.3 kDa and tagged *Yl*ADH2 40.8 kDa

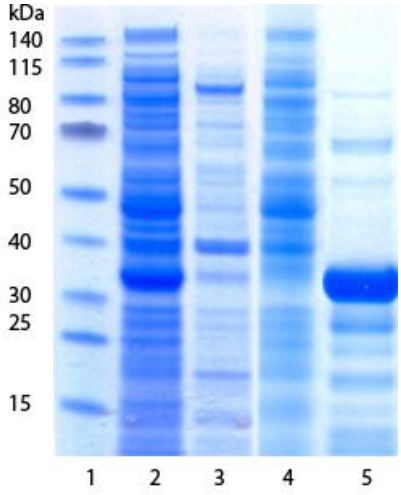



**Figure S3. NuPAGE gel of *Yl*HisSDR;** Lane 1: PageRuler Prestained Protein Ladder (Fermentas); Lane 2: pK470:*Yl*HisSDRcell free extract; Lane 3: pK470:*Yl*HTSDR cell debris; Lane 4: pK470:*Yl*HTSDR flow through from purification, Lane 4: purified *Yl*HTSDR; expected size of tagged *Yl*SDR is 31.9 kDa.

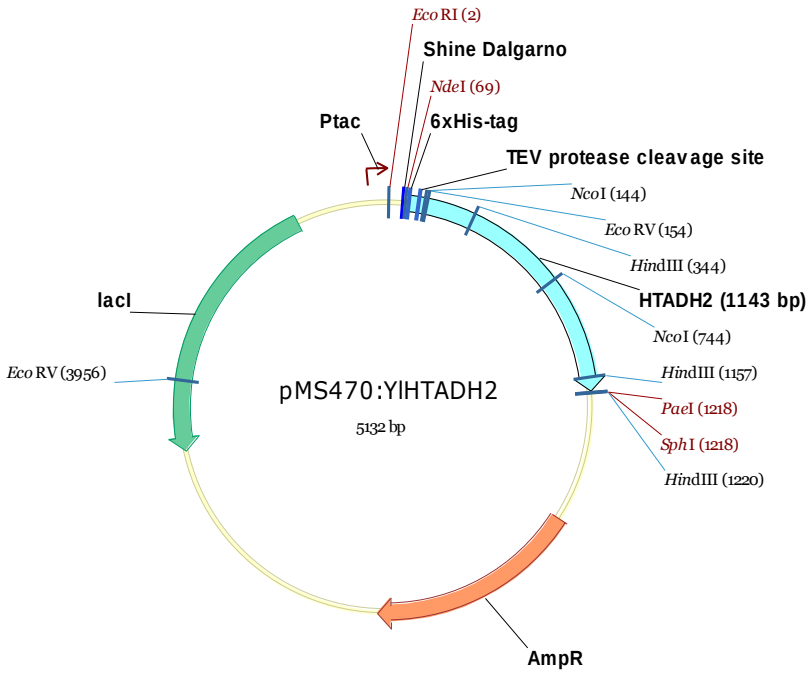



**Figure S4. pMS470:*Yl*HTADH2 vector map.** *P*tac – promoter *tac*, HTADH2– 6xHis-tagged alcohol dehydrogenase from *Y. lipolytica* CLIB 122, Amp$^r$ – fragment coding for ampicillin resitance. lacI – repressor *lac.*

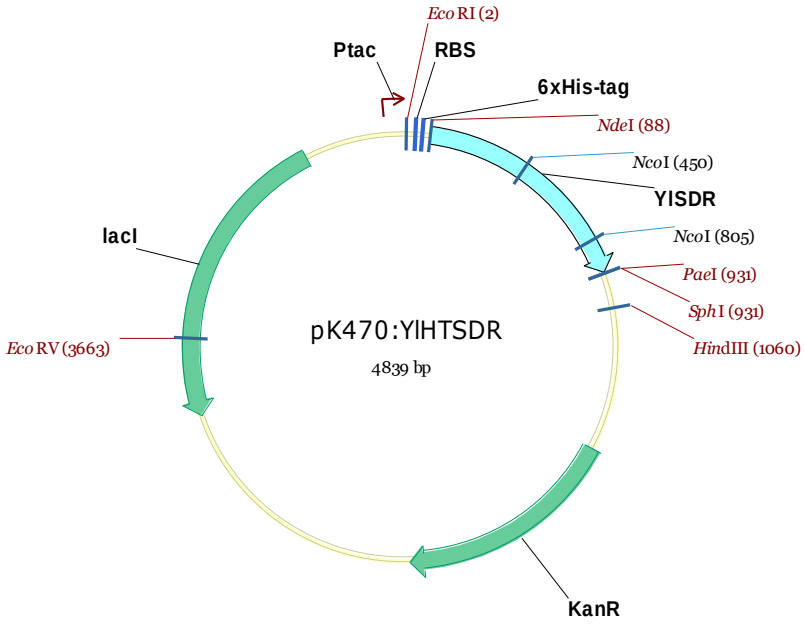



**Figure S5. pK470:*Yl*HTSDR vector map**, *P*tac – promoter *tac*, RBS – ribosome binding site, 6xHis-tag – polyhistidine-tag, *Yl*SDR – short chain dehydrogenase/reductase from *Y. lipolytica* CLIB 122, Kan$^r$ – fragment coding for kanamycin resitance, lacI – repressor *lac*

# 4 Aldehyde dehydrogenase from *Ralstonia eutropha* H16 (*Cupriavidus necato*r)

## 4.1 *Ralstonia eutropha* – an ubiquitous bacterium for special tasks

*Ralstonia eutropha* is a gram-negative, non-pathogenic bacterium ubiquitous in soil and water. It can use - alternatively or simultaneously - organic compounds as well as molecular hydrogen as an energy source (hence, it is also called a *Knallgas*–bacterium, which means hydrogen oxidizing bacteria), (Figure 15) [69].

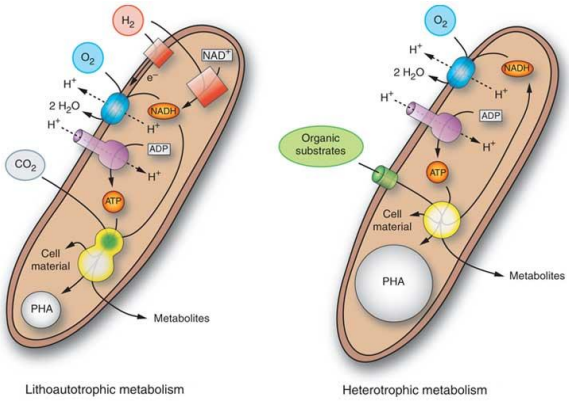



**Figure 15. Schematic representation of lithoautotrophic and heterotrophic metabolism in *R. eutropha.*** From Ref. [69].

Because *R. eutropha*'s has ability for degradation of chloroaromatic compounds and related pollutants, it is an important bioremediator. Moreover, as it can store organic carbon in polymer form of poly[(R)-3-hydroxybutyrate] (PHB) in specialized granules, it was applied for production of the biodegradable thermoplastic Biopol® [70]. Being able to use $CO_2$ as a sole carbon source, it has also gained the interest as producer of renewable liquid fuels – fatty acid derived methyl ketones [71]. Furthermore, an alcohol dehydrogenase from *Ralstonia* sp. DSM 6428 (*Ras*ADH) has been reported by Kroutil and co-workers to reduce space-demanding ketones, so called bulky-bulky ketones, like 1-phenyl-1-pentanone and 1-phenyl-1-hexanone [72]. Its preference for sterically demanding substrates was confirmed by the work of Kulig *et al.* [73]; and the recently published crystallographic studies of this enzyme revealed that it has a hydrophobic active site tunnel, which is well-suited for space-demanding substrates [74].

## 4.2 Reduction of 5-O-trityl-α,β-D-ribose with AlDH from *R. eutropha* H16

Knowing that *Ralstonia eutropha* and its enzymes exhibit strong reducing activity, we decided to investigate an aldehyde reductase EC 1.1.1.21 (*Ras*AlDH, GenBank: CAJ96944.1) for reduction of 5-*O*-trityl-α,β-D-ribose (5-*O*-TrR) – the same substrate, which was previously oxidized by *Yl*SDR [11] (section 2, p. 27). The *Ras*AlDH enzyme shares 28% identity, (82% query covery during BLASTp search) with the D-arabinose dehydrogenase 1 from *Saccharomyces cerevisiae* (Ara1, EC 1.1.1.117, encoded by YBR149W [75], pdb: 4ijc [76]). Ara1 was originally reported as one of two D-arabinose dehydrogenases taking part in biosynthesis of D-erythroascorbic acid (eAsA) from D-arabinose and showed oxidizing activity towards D-arabinose, L-xylose, L-fucose, and L-galactose (reduction was not tested) [75]. Although the role of Ara1 in eAsA biosynthesis was questioned by Amako *et al.* in 2006 [77,78], and the enzyme itself was finally annotated as α,β-dicarbonyl reductase belonging to the aldo-keto reductase family [79], its analog – *Ras*AlDH reduced 5-*O*-TrR with surprisingly good results (Section 4.4, pp. 59 – 62, Figure 18).

## 4.3 Experimental

### 4.3.1 General

Genomic DNA from *Ralstonia eutropha* H16 was was isolated according to the protocol of the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA) by Dr. Petra Köfinger. *E. coli* cells were cultivated in RS 306 and Multitron shakers (Infors AG), and the cells were harvested with an Avanti centrifuge J-20 (Beckman Coulter). Cell pellets were disrupted with a Branson 102C converter, power was supplied with a Branson Sonifier 250 and the cell free extract was obtained by centrifugation in an Ultracentrifuge Optima LE80K (Beckman Coulter). Enzymes were purified using a HisTrap™ FF 5 mL column on an ÄKTA Purifier 100 with Frac-950, software Unicorn 4.11, and desalted using a HiPrep™ 26/10 Desalting column on an ÄKTA Prime, software PrimeView 5.0 (GE Healthcare Life Sciences). Protein samples were analyzed with 4–12% SDS-PAGE® Bis-Tris gels. GDH was obtained from DSM Innovative Synthesis BV and 1 mg corresponds to 2.7 U[3] for NADPH oxidation. Antibiotics (kanamycin, ampicillin and gentamycin) and fromic acid were purchased from Sigma Aldrich/Fluka. 5-*O*-trityl-α,β-D-ribose was prepared as described previously [11] and obtained from Prof. Tanja Wrodnigg. Acetonitrile was purchased from J.T Baker, isopropyl β-D-1-thiogalactopyranoside (IPTG) from Biosynth, tryptic soy broth (TBS) from BD Bacto™, whereas NADPH (sodium salt; 98% pure), LB medium, LB-Agar (both Lennox) and all other chemicals and buffer components were purchased from Carl Roth and used as received. Nutrient broth (NB) consists of: peptone: 5g/L, yeast extract: 3 g/L and agar: 15 g/L and minimal salt media (MSM) was prepared as described in [80].

The HPLC analysis was carried out on an HPLC-MS (Agilent) using a Chromolith Performance RP-18 column with 1.2 mL/min, 60% of 0.1% formic acid and 40% acetonitrile as mobile phase. Detection was carried out with atmospheric pressure electrospray ionization, positive mode (MS) for scan (170-500) and 210 nm (UV).

[3] 1 unit of GDH catalyzes oxidation of 1 µmole of NADPH/min.

### 4.3.2 Construction of pEHisTEV:*Ras*AlDH

The fragment corresponding to *Ras*AlDH (dkgB, Gene ID: 4456032, GenBank: CAJ96944.1) was amplified from genomic DNA using Phusion® High-Fidelity DNA polymerase (Finnzymes) with the following primers: AraDHCnec_f: 5'- AAT TCC CAT GGA TGA GCG TTC CTT CAT TTG GTG TCG GG -3' and AraDHCnec_r: 5'- AAT TCT CGA GTC AGT CCC AGG CCG GGG CCA - 3'. The *Nco*I and *Not*I restriction sites are underlined. The purified PCR product was cloned into pEHisTEV vector previously digested with *Nco*I and *Not*I restriction sites and after sequence verification by LGC Genomic, transformed into electrocompetent *E. coli* BL21 (DE3) Gold cells (Stratagene). Because the overexpression of protein carried on pEHisTEV vector was not successful (probably because of the too strong T7 promoter), it was decided to re-clone the dkgB *gene* into the pMS470d8 vector containing the weaker tac promoter.

### 4.3.3 Construction of pMS470:*Ras*AlDH

Plasmid DNA of pEHisTEV:*Ras*AlDH was used as a template for amplification of *Ras*AlDH with following primers: AraDHCnec470_f: 5'- AAT CAC ATA TG AGC GTT CCT TCA TTT GGT GTC GGG ACT TTC -3'; AraDHCnec470_r: 5'- AAT CAG CAT GCT CAG TCC CAG GCC GGG GCC -3'. The *Nde*I and *Sph*I restriction sites are underlined. The purified PCR product was cloned into the pMS470 vector (Figure 16), previously digested with *Nde*I and *Sph*I and dephosphorylated with Calf Intestine Alkaline Phosphatase (Fermentas). The constructs were transformed into electrocompetent *E. coli* TOP10 F' cells (Invitrogen) and cells were plated out on LB with 100 µg/mL ampicillin (for pMS470). The plasmids were isolated with the GeneJET™ Plasmid Miniprep Kit (Fermentas) and the sequences confirmed by LGC genomics. The plasmids were then transformed into electrocompetent *E. coli* BL21 (DE3) Gold cells (Stratagene).

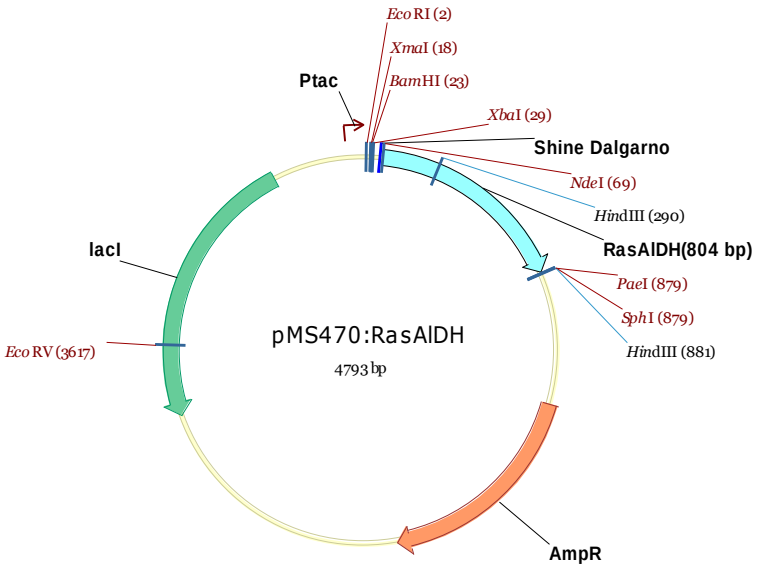



**Figure 16. pMS470:*Ras*AlDH vector map.** *P*tac – promoter *tac*, *Ras*AlDH – aldehyde dehydrogenase from *R. eutropha* H16, Amp$^r$ – fragment coding for ampicillin resitance. lacI – repressor *lac.*

### 4.3.4 Construction of pMS470:HT*Ras*AlDH[4]

Since re-cloning of His-tagged *Ras*AlDH directly from pEHisTEV vector into pMS470 failed because of unknown reasons, the pMS470:*Ras*ALDH was given to MSc. Zalina Magomedova, who used synthetic oligonucleotides coding for 6xHis-tag. For insertion of a 6xHistidine tag into the pMS470 expression vector two oligonucleotides were designed (6xHis sense: 5'- CTA GAA AGG AGA TAT ACC ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAC CA - 3' and 6xHis antisense: 5'- TAT GGT CGT AAT CGT GAT GGT GAT GGT GAT GGT AGT ACG ACA TGG TAT ATC TCC TTT – 3'). They contained an *Xba*I restriction site on the 5' end and an *Nde*I restriction site on the 3' end, for insertion to the amino terminus of the dehydrogenases respectively. To anneal the oligonucleotides and generate the linker, 2 nmol of each of them were mixed and incubated at 95 °C for 10 min. The mixture was then slowly cooled down to room temperature. The pMS470 plasmids containing the *Ras*AlDH was cut with *Xba*I and *Nde*I restriction enzymes and ligated with the annealed linker (Figure 17). Afterwards the constructs were transformed into competent *E. coli* TOP10 cells and named pMS470:HT*Ras*AlDH respectively and after positive verification it was re-transformed into *E. coli* BL21 (Novagene).

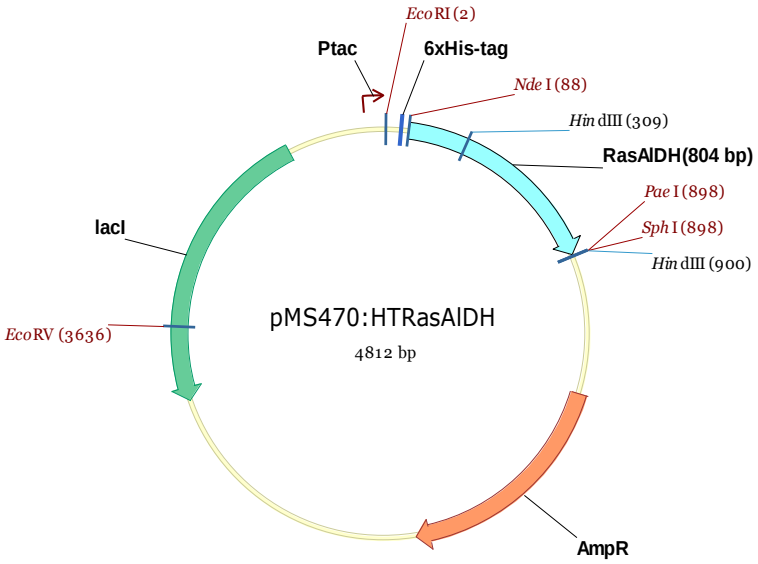



**Figure 17. pMS470:HT*Ras*AlDH vector map** *P*tac – promoter *tac*, 6xHis-tag – polyhistidine-tag, *Ras*AlDH – aldehyde dehydrogenase from *R. eutropha* H16, Amp$^r$ – fragment coding for ampicillin resitance, lacI – repressor *lac.*

[4] Work performed by MSc. Zalina Magomedova

### 4.3.5 Construction of pKRSF1010-Ptac:HT*Ras*AlDH and pKRep-Ptac:HT*Ras*AlDH[5]

For the expression in *R. eutropha,* HT*Ras*AlDH was cloned into plasmids, suitable for the transfer of DNA into *R. eutropha* H16. During conjugation DNA is transferred from a donor strain to a recipient strain. The pMS470:HT*Ras*AlDH construct was first linearized with *Sph*I, the ends were then blunted with the Fast DNA End Repair Kit (Thermo Scientific) and finally the *Ras*AlDH gene including a N-terminal 6xHis-tag was cut out with *Xba*I. The insert was then ligated into a pKRSF1010-Ptac and pKRep-Ptac [81]. Both vectors were previously digested with *Xba*I and *Hind*III, with the *Hind*III site being blunted as well. To improve ligation efficiency the backbone vectors were dephosphorylated with Fast Alkaline Phosphatase (Thermo Scientific). The constructs were transformed into competent *E. coli* TOP10 cells. The plasmids were again isolated from the received clones, cut with *Hind*III and *Spe*I (pKRep-Ptac:HTRasAlDH) or *Hind*III *and EcoR*V (pKRSF1010-Ptac:HTRasAlDH) and analyzed on a 1% agarose gel.

### 4.3.6 Expression and purification of HT*Ras*AlDH

#### 4.3.6.1 Heterologous expression and purification of HT*Ras*AlDH[4]

*E. coli* BL21 harboring HT*Ras*AlDH plasmids were cultivated as follows: overnight cultures (30 mL LB with 100 µg/mL ampicillin were inoculated with a single colony and grown overnight at 37 °C in an orbital shaker at 110 rpm. 400 mL LB medium with 100 µg/mL ampicillin in 2 L baffled Erlenmeyer flasks were inoculated to an $OD_{600}$ of 0.05. These main cultures were grown for 3.5 h at 37 °C and 90 rpm to an $OD_{600}$ of 1.4 and induced with 0.1 mM IPTG. The cultures were incubated for 24.5 h at 37 °C and 90 rpm. The cells were harvested by centrifugation (2,831 x *g*, 4 °C, 20 min), washed with Tris-HCl buffer (50 mM, pH 7.5), and stored overnight at -20 °C or disrupted by sonication. After centrifugation at 72,647 x *g*, 4 °C for 1 h, the cell free extract was subjected to Ni-affinity chromatography, re-buffered 20 mM Tris-Hcl, 200 mM NaCl, pH 7.5, concentrated with Vivaspin 20 (Sartorius Stedim Biotech S.A), and stored at +4 °C. Protein concentrations were determined using the NanoDrop.

#### 4.3.6.2 Homologous expression of HT*Ras*AlDH in *Ralstonia eutropha* H16 under heterotrophic and lithoautotrophic conditions[5]

*E. coli* S17-1 cells were grown at 37 °C in LB medium. *R. eutropha* H16 cells were grown at 28 °C in tryptic soy broth (TSB) with 0.6% fructose. Antibiotics: kanamycin (40 µg/mL for *E. coli*; 200 µg/mL for *R. eutropha* harboring plasmids) and gentamycin (20 µg/mL for *R. eutropha*) were added to the media as appropriate.

The plasmids pKRSF1010-Ptac:HT*Ras*AlDH and pKRep-Ptac:HT*Ras*AlDH were transformed into electrocompetent *E. coli* S17-1 cells (donor strain), which contain chromosomally integrated *tra* genes needed for conjugation. The transfer of the plasmids into *R. eutropha* H16 was performed conjugative via "agar-spot-mating", previously described by Friedrich *et al.* [82]. Therefore, the donor and recipient strain were grown overnight and the cells were then harvested by centrifugation (2,831 x *g*, 4 °C, 20 min) and resuspended in sterile 0.9% NaCl. Recipient and donor cells were mixed

[5] Work performed by BSc. Eva Thaler

at a ratio of 1:1 and 400 µL of this mixture were spotted on nutrient broth (NB) plates. After incubation at 28 °C for 20 h the cells were washed off the agar with 3 mL 0.9% NaCl. A diluted portion, equivalent to 10 µL of the cell suspension was plated out on selective TSB plates. Transconjugants appeared after 2-3 days of incubation at 28 °C.

Heterotrophic cultivation of *R. eutropha* H16 pKRSF1010-Ptac:HT*Ras*AlDH and pKRep-Ptac:HT*Ras*AlDH was set up as follows: overnight cultures were inoculated with a single colony and grown overnight at 28 °C in an orbital shaker at 110 rpm. 2 L baffled Erlenmeyer flasks containing 300 mL TSB media with 0.6% fructose and the appropriate antibiotic were inoculated to an $OD_{600}$ of 0.1. These main cultures were grown at 28 °C and 90 rpm for 28 h. The cells were harvested by centrifugation (2,831 × *g*, 4 °C, 20 min), washed with Tris-HCl buffer (50 mM, pH 7.5) and stored ON at −20 °C.

*R. eutropha* H16 pKRSF1010-Ptac:HT*Ras*AlDH and pKRep-Ptac:HT*Ras*AlDH were lithoautotrophically cultivated in minimal salt media (MSM) as described by Schlegel *et al.* [83]. The gas atmosphere for autotrophic growth contained a mixture of 80% hydrogen, 10% oxygen, and 10% carbon dioxide (v/v). The cultivation was set up as follows: heterologous overnight cultures (25 mL TSB with 0.6% fructose and the appropriate antibiotics) were inoculated with a single colony and grown overnight at 28 °C in an orbital shaker at 110 rpm. The next day the cells were harvested, washed twice and then resuspended in 0.9% sterile NaCl. In a pre-culture (25 mL MSM; 2-3 days, 28 °C, 90 rpm) the cells adapt to the lithoautotrophic metabolism. Subsequently, 250 mL MSM media containing the appropriate antibiotic were inoculated with the pre-culture and the main cultures were grown for 2-3 days at 90 rpm. The cells were harvested by centrifugation (2,831 × *g*, 4 °C, 20 min), washed with Tris-HCl buffer (50 mM, pH 7.5) and stored ON at -20°C.

### 4.3.4 Reduction of 5-*O*-trityl-α,β-D-ribose with AlDH from *R. eutropha* H16

The reduction of 5-*O*-trityl-α,β-D-ribose (5-*O*-TrR) – a bulky and lipophilic substrate, whose preparation and oxidation was described previously [11] (section 2, p. 27)was examined both with purified enzyme HT*Ras*AlDH as well as with whole *E. coli* and *R. eutropha* cells, overexpressing the enzyme hetero- and homologously, or having the respective empty vector control (EVC), (Figure 19). Typically, 0.065 and 0.13 mg of purified enzyme or 50 – 200 mg of thawed whole cells were dispersed in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM of NADPH (or alternatively 0.5 mM NADPH, 0.135 or 0.27 U GDH and 100 mM of glucose for cofactor recycling). The experiments without cofactor addition were also prepared in case of whole cell bioconversion. The reactions were started by addition of 25 µL of 5-*O*-TrR (100 mM in DMSO) to give final concentrations of 5 mM substrate and 5% v/v DMSO. The reactions proceeded in 1.5 mL reaction tubes at 28 °C in an Eppendorf Thermomixer at 900 rpm for 21-24 h (buffer, pH and temperature were determined previously as optimal conditions for reductions carried out with *Ras*AlDH[6]). All reactions were carried out in triplicates, except the experiment conducted with *R. eutropha* pKRSF1010-Ptac:HT*Ras*AlDH cells from lithoautotrophic growth. These reactions were carried out as duplicates, because of small amount of cells. The bioconversion reactions were stopped by addition of 500 µL of acetonitrile. After centrifugation, the supernatants were analyzed by HPLC.

[6] Magomedova, Z. (2013). Personal communication

## 4.4 Results and discussion

In the conducted experiment, 5-*O*-trityl-α,β-D-ribose (5-*O*-TrR) was used as a substrate for reduction with whole cells as well as with purified HT*Ras*AlDH.

The results of the experiments with the purified enzyme showed that HT*Ras*AlDH can reduce 5-*O*-TrR to the respective alcohol in almost 80% (in the presence of double excess of cofactor). Usage of double amount of enzyme increased the conversion by 20%, and application of the GDH/glucose cofactor recycling system resulted in almost 70% of product after overnight reaction (black bars on Figure 18).

The results of reduction catalyzed by *E. coli* cells (grey and white bars on Figure 18) are questionable. On the one hand they suggest that even the *E. coli* cells carrying EVC are active for reduction of 5-*O*-TrR to the respective alcohol (white bars), but on the other hand it has to be stressed that in case of all *E. coli* whole cell bioconversions the mass balance problem had appeared: The detected amount of substrate and product was sometimes only 25% of the expected value. This indicates a substrate/product loss or decomposition within this system and because of that the presented values are not trustworthy.

Quite different is the situation in the case of homologous overexpression of HT*Ras*AlDH (blue and green bars on Figure 18). The conducted experiments clearly showed that the strains carrying additional copies of dkgB genes have higher reduction activity towards 5-*O*-TrR than the cells carrying EVC. Surprisingly, all *R. eutropha* cells cultivated heterotrophically, showed very similar activity and reduced around 85% of 5-*O*-TrR after overnight reaction, regardless of the presence or absence of cofactor or cofactor recycling system (Figure 18, dark green and dark blue bars representing *R. eutropha* carrying pKRSF1010-Ptac:HT*Ras*AlDH and pKRep-Ptac:HT*Ra*sAlDH plasmids, respectively). It cannot be excluded that the reduction is in fact higher, but because of an internal cofactor recycling system some part of $NADP^+$ takes part in the re-oxidation of the formed alcohol back to the ribose. Some differences in the cells' activity are only visible when the reaction is stopped after 4h (Figure 18, light green and light blue bars). After this time the reaction was fastest in case of *R. eutropha* pKRSF1010-Ptac:HT*Ras*AlDH in the presence of GDH/glucose (74 ± 3%), followed by *R. eutropha* pKRep-Ptac:HT*Ra*sAlDH in the presence of double excess of cofactor (72 ± 2.8%). The lowest conversion was gained with *R. eutropha* pKRSF1010-Ptac:HT*Ras*AlDH without any cofactor addition and cofactor recycling system and reducing 45 ± 0.2% of 5-*O*-TrR to the alcohol within 4h.

The experiment with lithoautotrophically grown *R. eutropha* cells showed that *R. eutropha* pKRSF1010-Ptac:HT*Ras*AlDH cells converted 90 ± 0.3% of 5-*O*-TrR in the presence of 10 mM NADPH and only little less (84 ± 3.1%) in the presence of the GDH/glucose. Unfortunately, the avaliable amount of cells was not sufficient to conduct both experimental set ups (4h and ON), hence, just the overnight experiment was conducted. In contracts to cells carrying the plasmid pKRSF1010-Ptac:HT*Ras*AlDH, cells carrying the pKRep-Ptac:HT*Ra*sAlDH plasmid and showed surprisingly low conversion (40 ± 1.6% in the best case after overnight reaction, Figure 18, green-hatched bars). One reason therefore can be the considerably lower expression level of HT*Ra*sAlDH in *R. eutropha* H16 pKRep-Ptac than from pKRSF1010-Ptac in lithoautotrophic conditions, as shown in Figure 19. The lower amount of expressed enzyme can be caused by the prolonged cultivation time, which was intended to increase the total cell yield. Six days cultivation of *R. eutropha* H16 pKRep-

Ptac:HT*Ras*AlDH in comparison to two 2.5 days for *R. eutropha* H16 pKRSF1010-Ptac:HT*Ras*AlDH could also lead to an increased level of proteases, which, in turn, could have caused the degradation of *Ras*AlDH.

The general lower level of bioconversion without any cofactor addition, obtained with *R. eutropha* pKRSF1010-Ptac:HT*Ras*AlDH cells, which were grown lithoautotrophically in comparison to cells grown heterotrophically (62 ± 0.4% and 85 ± 0.9% after overnight reaction, respectively), indicates that the reduction equivalents under the lithoautotrophic growth conditions were used up during growth of the cells. This could be caused by the internal isocitrate dehydrogenase, which is up-regulated in such growth conditions [84]. To omit this limitation, the next step would be the addition of substrate to growing cells and the monitoring of its consumption and product formation during the time, as it was reported by Oda *et al.* [85].

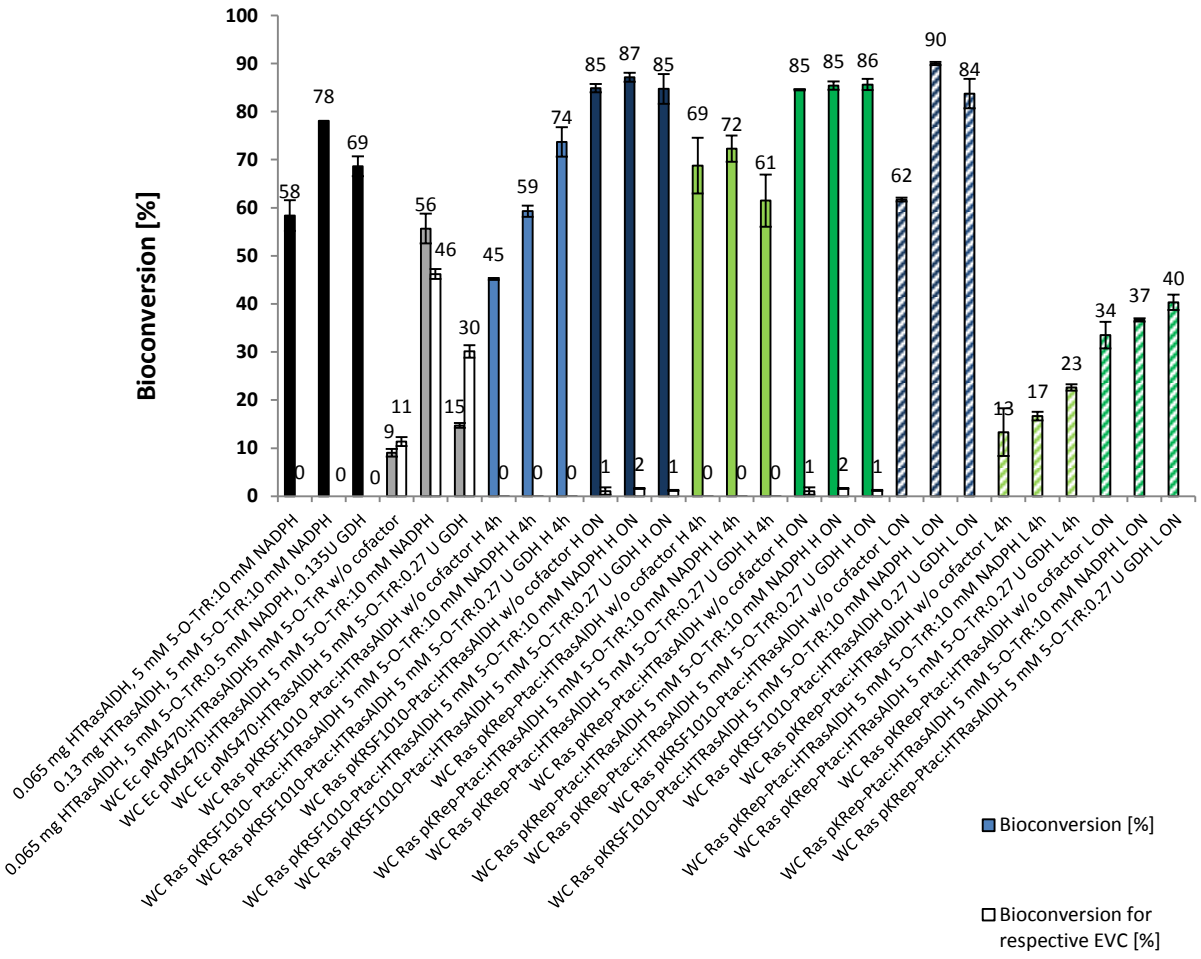
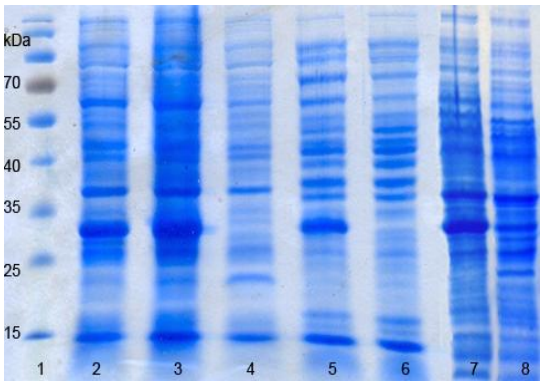



**Figure 18. Reduction of 5-*O*-trityl-α,β-D-ribose to the respective alcohol with purified HT*Ras*AlDH and with whole *E. coli* and *R. eutropha* cells, expressing *Ras*AlDH hetero- and homologously, respectively.** WC Ec – whole *E. coli* cells, WC Ras – whole *R. eutropha* H16 cells, GDH – glucose dehydrogenase, U – enzyme unit, H – heterotrophically cultivated cells, L – lithoautotrophically cultivated cells.




| | |
|---|---|
| **1** | PageRuler Prestained Protein Ladder from Thermo Scientific |
| **2** | *R.eutropha* pKRSF1010-Ptac:HT*Ras*AlDH cultivated heterotrophically |
| **3** | *R.eutropha* pKRepPtac:HT*Ras*AlDH cultivated heterotrophically |
| **4** | *R.eutropha* pKRSF1010-Ptac |
| **5** | *E. coli* BL21 pMS470:HT*Ras*AlDH |
| **6** | *E. coli* BL21 pMS470d8 |
| **7** | *R.eutropha* pKRSF1010-Ptac:HT*Ras*AlDH cultivated lithoautotrophically for 2.5 days |
| **8** | *R.eutropha* pKRepPtac:HT*Ras*AlDH cultivated lithoautotrophically for 6 days. |

**Figure 19. SDS-PAGE of cell free extracts of *E. coli* and *R. eutropha* cells (both overexpressing HT*Ras*AlDH as well as empty vector controls). MW of HT*Ras*AlDH: 30,47 kDa.** Running buffer: 25 mM Tris, 190 mM glycine, 0.1% SDS; pH 8.6.

## 4.5 Conclusions

The obtained results complement previously reported experiments relying on the oxidation of 5-*O*-TrR to the respective lactone with *Yl*SDR [11]. With these two enzymes: *Yl*SDR and *Ras*AlDH it is possible to catalyze both the reduction as well as the oxidation of 5-*O*-TrR in more than 79% (Figure 20). Hence, the next step in the usage of oxidoreductases for proceeding of bulky substrates has been done.

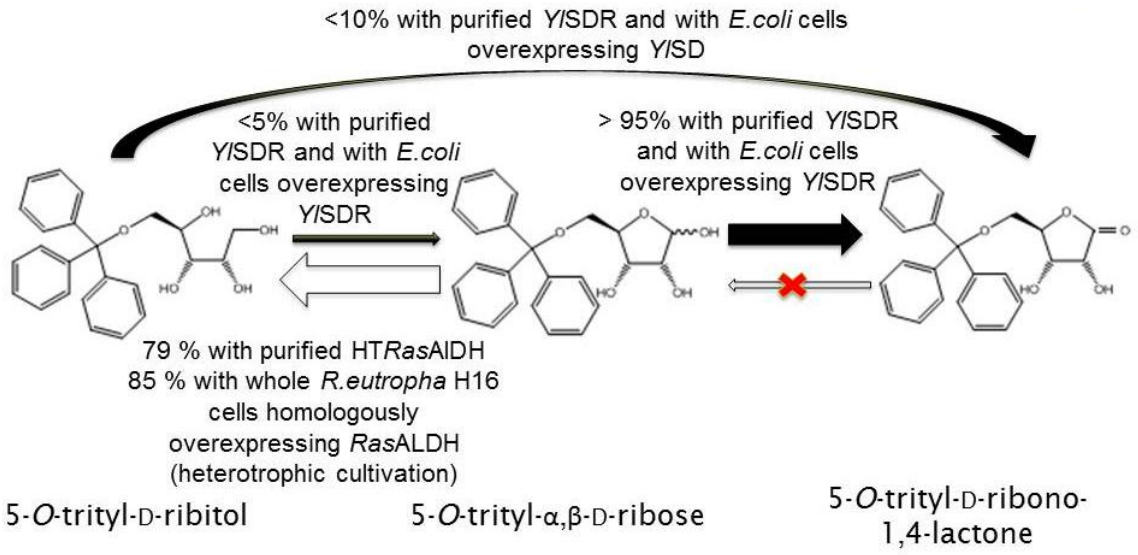



**Figure 20. Oxidation and reduction of 5-*O*-trityl-α,β-D-ribose to the respective lactone and alcohol catalyzed by *Yl*SDR and *Ras*AlDH, respectively.**

# 5 Biocatalytic reduction of carboxylic acids

**Biotechnology Journal**

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**Biocatalytic reduction of Carboxylic Acids**

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Review

# Biocatalytic reduction of carboxylic acids

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An increasing demand on non-petroleum based products is envisaged in the near future. Carboxylic acids such as citric acid, succinic acid, fatty acids and many others are available in abundance from renewable resources and they could serve as economic precursors for bio-based products such as polymers, aldehyde building blocks and alcohols. However, we are confronted with the problem that carboxylic acid reduction requires a high level of activation due to the carboxylate's thermodynamic stability. Catalytic processes are scarce and often, their chemoselectivity is insufficient. This review points at bio-alternatives: currently known enzyme classes and organisms that catalyze the reduction of carboxylic acids are summarized.

**Keywords:** Carboxylate reductase * α-Aminoadipate reductase * Aryl-aldehyde dehydrogenase ($NADP^+$)* Carboxylic acid * Aldehyde * Alcohol

**A list of abbreviations:**

| | |
|---|---|
| AOR | aldehyde ferrodoxin oxidoreductase |
| CAR | carboxylic acid reductase |
| CAV | 1,1'-carbamoyl methyl-viologen |
| MV | methyl viologen |
| PPTase | phosphopantetheinetransferase |
| RTP | red-colored tungsten-containing protein |
| TMV | 1,1',2,2'-tetramethylviologen |

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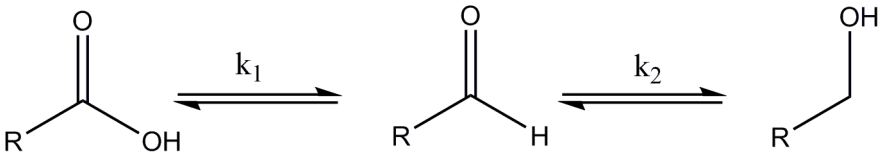
## 1 Introduction

Carboxylic acids are available in great abundance from renewable sources: e.g. formic acid, acetic acid, citric acid, oxalic acid, valeric acid, succinic acid, palmitic acid, arachidic acid, ferulic acid and many others. Succinic acid, for example, can serve as precursor for the production of bio-based polymers and industry is currently implementing new plants for its production [1]. Also synthetic fatty acids are interesting from the industrial point of view, because they can serve as source for fatty alcohols production, which in turn can be used as biofiuels, components for production detergents, urfactants and polymers.[2]. Increasingly cheaper carboxylic acid production, especially from renewable sources [3], makes them attractive substrates for a whole range of alternative products. The ultimate goal should be to supplement traditional mineral oil-based intermediates with sustainable technologies. However, to access aldehydes and alcohols from carboxylic acids, efficient reductive processes will become necessary. Industry is met with the problem that carboxylic acid reduction demands strong reduction power.

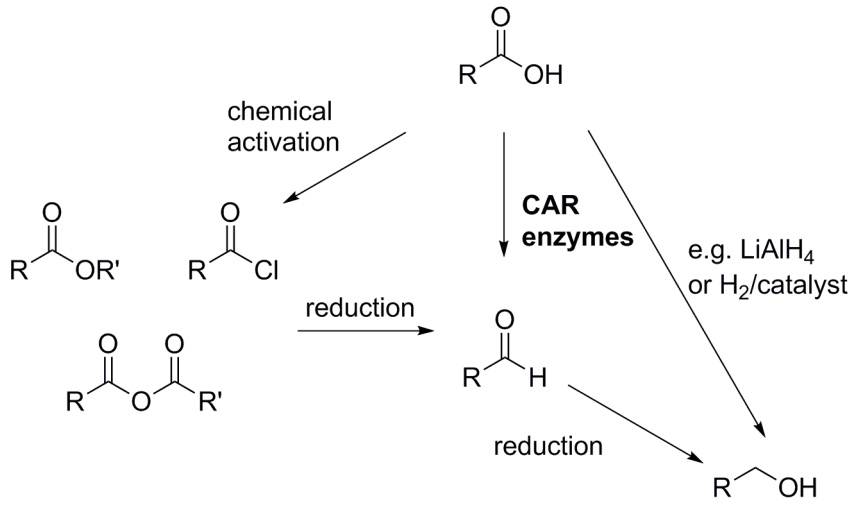
The carbon atom in the COOH moiety is present at its highest possible oxidation state apart from carbon dioxide and tetrahalogenated carbons. From the thermodynamic viewpoint, this functional group is in an energetically favored state. Consequently, it shows little reactivity and needs a high level of activation in order to participate in chemical reactions. Once this energetic barrier is overcome, the reduction formally yields the respective aldehyde in the first step, which is more reactive than the carboxylic acid (Scheme 1). Therefore, the aldehyde does not accumulate but is reduced further to the respective primary alcohol. In case the desired product is the aldehyde, appropriate measures need to be taken that prevent this over-reduction. A number of examples for chemical reductions of carboxylic acids to the corresponding aldehydes were reported in the literature (Scheme 2) [4]. However, the routinely used strategy is based on the overreduction to the alcohol and subsequent selective

re-oxidation to the aldehyde with the aid of pyridinium chlorochromate, Dess-Martin periodinane, TEMPO or via Swern oxidation [5]. Most of these methods bear in common that they are either expensive or use environmentally harmful reagents.




**Scheme 1**. **Reduction of carboxylic acids (k1 << k2)**

The vast majority of chemical reductions of carboxylic acids proceed via the formation of activated derivatives. These may e.g. be esters or amides, acyl halogenides, anhydrides or carbonates [6,7]. Typically, complex metal hydrides such as lithium aluminum hydride and sodium borohydride or variants thereof are used in stoichiometric amounts [8,9]. Catalytic hydrogenations are frequently performed by heterogeneous metal catalysts, however, also homogenous catalytic hydrogenations are strongly emerging [10]. In the past two decades, several selective chemical reductions to alcohols or aldehydes using hydrosilanes have been described [11].




**Scheme 2.** **Overview of strategies for the reduction of carboxylic acids**

Today, producers of pharmaceuticals and fine chemicals appreciate enzymatic reactions in general, and also biocatalytic reduction processes are gaining importance [12]. In this review, we are giving a comprehensive overview of biocatalytic reductions of carboxylic acids.

Enzyme catalyzed carboxylic acid/carboxylate reduction is a relatively young field in comparison to the extensively studied and applied enzymatic carbonyl reduction. Like the latter, it offers the typical advantages of biotransformation processes:

- Mild conditions: it proceeds in an aqueous environment at ambient conditions (room temperature, atmospheric pressure)

- Chemoselectivity: other reducible moieties remain unaffected
- Enantioselectivity: enzymes distinguish enantiomers

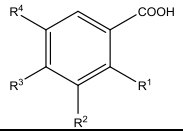
The following chapters summarize the reduction of carboxylic acids by the application of biocatalysts. In most cases, the applied catalyst consisted of cells rather than isolated enzymes and more often than not, the acids were converted to mixtures of aldehyde and alcohol, assumingly due to background reactions mediated by the respective environment of the enzyme.

To give an idea, which compounds may be converted enzymatically, Tables 1-5 provide an overview reported substrates. Note that several publications also include lists of non-substrates, which were omitted in Tables 1-5 for clarity.

## 2 Aerobic carboxylate reductions by fungi

To our knowledge, the first report of a biocatalyzed carboxylate reduction appeared in 1959 in which the white-rot fungus of the division Basidiomycota (*Trametes versicolor*, also known as *Polystictus versicolor, Polyporus versicolor, Coriolus versicolor* and several other names) reduced a small number of aromatic carboxylic acids (Table 1, entries: 1, 20-23, 27) as well as 2-naphthoic acid (Table 4, entry 2) to the respective aldehydes and further to the alcohols [13,14]. Further examples of chloro- and nitrobenzoic acids reduction by *T. versicolor* IFO 4937 as well as aminobenzoic, nitrophenylacetic and chlorocinnamic acid by *T. versicolor* IFO 4941 to the respective alcohols followed [15]. Another species from the genus *Trametes*, namely *T. hirsutus* IFO 4917 reduced 3,4,5-trimethoxybenzoic, 3,4-dimethoxycinnamic and 2-methoxyphenylacetic acid to the respective alcohols [15]. *Psilocybe zapotekorum* DSM 1891 reduced ferulic acid to coniferyl alcohol. Interestingly, no reduction activity towards benzoic and phenylacetic acid and its hydroxyl derivatives was found in this study [15], in contrast to reports by Farmer [13] and Nishida [16]. This may be an indication for significant differences of carboxylate reductase expression within one species. In addition to the reduction of ferulic acid to the corresponding aldehyde [17] and alcohol [16], also vanillic acid, vanillin and vanillyl alcohol were observed as metabolites in *Trametes* sp. and *Fomes fomentarius* [17]. This observation gave rise to detailed investigations in several research groups with the aim to generate effective microbial vanillin producers. Similar ferulic acid metabolism was reported in other fungi such as *Sporotrichum pulverulentum* ATCC 32629 [18] and *Pycnoporus cinnabarinus* I-937 [19]. This latter species has been in the limelight of this group and in further experiments they combined two filamentous fungi: *Aspergillus niger* I-1472 was used in a process for the transformation of ferulic acid to vanillic acid, which, in turn, was reduced to vanillin by *P. cinnabarinus* MUCL 39532 [20,21]. Several aspects of this strategy towards vanillin production were subsequently studied [22–24] and applied [25,26]. Furthermore, also *Phanerochaete chrysosporium* (another member of Polyporales) was used as a reducing agent both in two step [27,28] as well as in a one-step vanillin production procedure [29].

**Table 1. Benzoic acid derivatives reported as substrates for carboxylate reductases**




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | Biocatalyst | Ref |
|---|---|---|---|---|---|---|
| 1 | H | H | H | H | *Trametes versicolor* mycelium | [13] |
| | | | | | *Aspergillus niger* Perlman Wisconsin 72-4 mycelium | [34] |
| | | | | | *Nocardia asteroids* JCM 3016, *Rhizopus oryzae* IFO 5440 and IFO 4706, *Mucor fragilis* IFO 6449 and *Mucor javanicus* IFO 4569 and *Homoconis resinae* F328 resting cells | [35,36] |
| | | | | | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 resting cells, crude extract, purified CAR from it and from heterologous overexpression in *E. coli* [a)]; also *E. coli* resting cells heterologously overexpressing *Ni*CAR | [46–48,50] |
| | | | | | *Clostridium thermoaceticum* resting cells[b)] | [55] |
| | | | | | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| | | | | | *Clostridium formicoaceticum* DSM 92 crude extract[d)] | [81] |
| | | | | | Purified W-AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | *Actinomyces* sp.[f)] | [62] |
| | | | | | *Pyrococcus furiosus* DSM 3638, growing or resting cells | [70,71] |
| 2 | Cl | H | H | H | *Trametes versicolor* IFO 4937 mycelium | [15] |
| | | | | | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 3 | H | Cl | H | H | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| | | | | | *Desulfomicrobium escambiense* growing cells | [63] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells and cell free extracts supplemented with electron carrier and donor | [64] |
| | | | | | Purified CAR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| 4 | H | Cl | OMe | H | *Bjerkandera* sp. strain BOS55 mycelium | [31] |
| 5 | Cl | H | Cl | H | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 6 | Cl | H | H | Cl | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 7 | H | Cl | Cl | H | *Bjerkandera* sp. strain BOS55 mycelium | [31] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 8 | H | Cl | H | Cl | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 9 | H | Cl | OMe | Cl | *Bjerkandera* sp. strain BOS55 mycelium | [31] |
| 10 | H | H | Cl | H | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | *Clostridium thermoaceticum* resting cells[b)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| | | | | | Purified CAR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 11 | Br | H | H | H | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 12 | H | Br | H | H | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | *Desulfomicrobium escambiense* growing cells | [63] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 13 | H | H | Br | H | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 14 | H | I | H | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 15 | H | H | I | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| 16 | F | H | H | H | *Bjerkandera adusta* growing cells | [30] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 17 | F | H | H | Me | *Bjerkandera adusta* growing cells | [30] |
| 18 | H | F | H | H | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| | | | | | Purified W-AOR and Mo-AOR from *Clostridium* | [57,60] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | *formicoaceticum* DSM 92[c)] | |
| | | | | | *Bjerkandera adusta* growing cells | [30] |
| | | | | | *Nocardia asteroides* JCM 3102 resting cells | [35,36] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 19 | H | H | F | H | *Bjerkandera adusta* growing cells | [30] |
| | | | | | *Bjerkandera* sp. strain BOS55 mycelium | [31] |
| | | | | | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| | | | | | Purified CAR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | *Clostridium thermoaceticum* resting cells DSM 521[b)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 20 | OMe | H | H | H | *Trametes versicolor* mycelium | [13] |
| | | | | | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 21 | OMe | H | OMe | H | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| 22 | H | OMe | H | H | *Trametes versicolor* mycelium | [13,14] |
| | | | | | *Aspergillus niger* ATCC 9142 and *Corynespora cassicola* IFO 7483 mycelium | [15] |
| | | | | | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | | | | Purified W-AOR and Mo-AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57,60] |
| | | | | | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 23 | H | OMe | OMe | H | *Trametes versicolor* mycelium | [13] |
| | | | | | *Bjerkandera* sp. strain BOS55 mycelium | [31] |
| | | | | | *Actinomyces* sp.[f)] | [62] |
| 24 | H | OMe | OH | H | *Trametes* sp. mycelium | [16] |
| | | | | | *Sporotrichum pulverulentum* ATCC 32629 mycelium | [18] |
| | | | | | *Pycnoporus cinnabarinus* I-937, MUCL 39532 and SW-0204 mycelium | [19–26] |
| | | | | | *Phanerochaete chrysosporium* ATCC 24725 and MIC 247 mycelium | [27–29] |
| | | | | | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 resting cells, crude extract, [a)] purified CAR isolated from it and from heterologous overexpression in *E. coli*[a)] | [42,47,50] |
| | | | | | *Schizosyccharomyces pombe* and *Saccharomyces cerevisiae* growing cells | [51] |
| | | | | | *Actinomyces* sp.[f)] | [62] |
| | | | | | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| 25 | H | OMe | OH | OMe | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| | | | | | *Actinomyces* sp.[f)] | [62] |
| 26 | H | OMe | OMe | OMe | *Trametes hirsutus* IFO 4917 mycelium | [15] |
| | | | | | *Actinomyces* sp.[f)] | [62] |
| 27 | H | H | OMe | H | *Trametes versicolor* mycelium | [13] |
| | | | | | *Corynespora cassicola* IFO 7483 mycelium | [15] |
| | | | | | *Bjerkandera* sp. strain BOS55, *Dichomitus squalens* CBS 432.34, *Phlebia tremellosa* ATCC 60027, *Phlebia brevispora* KBT 89, *Phenerochaete chrysosporium* ATCC 24725, *Schizophyllum commune* PW 94.3, *Trametes hirsura* CBS 282.73, *Trametes versicolor* 290, *Trametes gibbosa* RHEN 93.2, *Pleurotus eryngii* CBS 613.91, *Stereum hirsutum* PW 93.4, *Lentinus tigrinus* PN 94.2 and *Polporus cilatus* ONO 94.1 mycelium | [31] |
| | | | | | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | | | | *Actinomyces* sp.[f)] | [62] |
| | | | | | Purified AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | *Clostridium thermoaceticum* resting cells DSM 521[b)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 28 | OH | H | H | H | *Neurospora crassa* cell free extract and purified CAR[a)] | [33,45] |

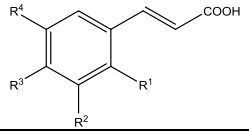
| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | *Aspergillus niger* Perlman Wisconsin 72-4 mycelium | [34] |
| 29 | H | OH | H | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | | | | Purified W-AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 30 | H | OH | OH | H | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| 31 | H | H | OH | H | *Aspergillus niger* mycelium | [34] |
| | | | | | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Fomes fomentarius* mycelium | [17] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | | | | Purified W-AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | *Clostridium thermoaceticum* resting cells DSM 521[b)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 32 | H | H | OPh | H | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 33 | $NO_2$ | H | Cl | H | *Hebeloma sacchariolens* fruit bodies and homogenate | [32] |
| 34 | H | $NO_2$ | H | H | *Trametes versicolor* IFO 4937 mycelium | [15] |
| | | | | | *Bjerkandera* sp. strain BOS55 mycelium | [31] |
| 35 | H | H | $NO_2$ | H | *Trametes versicolor* IFO 4937 mycelium | [15] |
| 36 | $NH_2$ | H | H | H | *Hebeloma sacchariolens* fruit bodies | [32] |
| | | | | | *Aspergillus niger* Perlman Wisconsin 72-4 mycelium | [34] |
| 37 | $NH_2$ | H | F | H | *Hebeloma sacchariolens* fruit bodies and homogenate | [32] |
| 38 | $NH_2$ | H | H | F | *Hebeloma sacchariolens* fruit bodies and homogenate | [32] |
| 39 | H | $NH_2$ | H | H | *Trametes versicolor* IFO 4941 mycelium | [15] |
| | | | | | *Desufovibrio vulgaris* PY1 growing cells | [64] |
| 40 | H | $NH_2$ | OH | H | *Streptomyces griseus* GriC/GriD purified | [79] |
| 41 | H | H | $NH_2$ | H | *Aspergillus niger* Perlman Wisconsin 72-4 mycelium | [34] |
| 42 | H | H | AcNH | H | *Nocardia iowensis* sp. NRRL 5646 crude extract | [46] |
| 43 | Me | H | H | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract | [46] |
| 44 | H | Me | H | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | Purified W-AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 45 | H | H | Me | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | Purified AOR from *Clostridium formicoaceticum* DSM 92[e)] | [57] |
| | | | | | *Clostridium thermoaceticum* resting cells DSM 521[b)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 46 | Ac | H | H | H | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 47 | H | H | Ac | H | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 48 | Ph | H | H | H | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 49 | H | H | Ph | H | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 50 | COOH | H | H | H | *Neurospora crassa* SY7A purified CAR[a)] | [45] |
| 51 | H | H | $CH_2OH$ | H | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |
| 52 | H | H | COOH | H | *Clostridium thermoaceticum* DSM 521 resting cells[b)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[c)] | [59] |

[a)] In the presence of ATP and NADPH; [b)] In the presence of CO and MV; [c)] In the presence of $CAV^{+\bullet}$; [d)] In the presence of CO;[e)] In the presence of $TMV^{+\bullet}$; [f)] Lyophilized and permeabilized whole cells

The reduction of benzoic acid derivatives was also reported from other Basidiomycota, especially from the genus *Bjerkandera*. All investigated fluorinated benzoic acid substrates (Table 1, entries: 16-19) were metabolized by *Bjerkandera adusta* to the corresponding aldehydes. Interestingly, only the 2-fluorinated derivative was further reduced to the corresponding alcohol [30]. Hage et al. studied the tolerance of different fungi for high concentrations of aryl acids (10 mM) and screened them for the reduction of 4-anisic acid. In

course of these studies, 13 fungal strains from different genera were identified with this activity, and typically, a product mixture of aldehyde and alcohol was found. *P. brevispora* KBT 89, by contrast, fully reduced all aldehyde to 4-anisyl alcohol as a product (Table 1, entry 27). *Bjerkandera* sp. strain BOS55 not only reduced 4-anisic acid but also a number of other substrates such as halogenated benzoic acid derivatives (Table 1, entries: 4,7,9,19) [31]. Amine containing benzoic acid derivatives (anthranilic acid and related compounds including *N*-heterocyclic structures) were also subjected to Basidiomycota metabolism and *Hebeloma sacchariolens* was identified as a biocatalyst for reductive transformations of this compound class [32].

**Table 2. Cinnamic acid derivatives as substrates for carboxylate reducing activities**



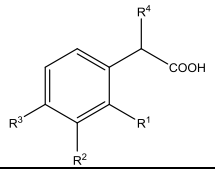

| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | Biocatalyst | Ref |
|---|---|---|---|---|---|---|
| 1 | H | H | H | H | *Corynespora cassicola* IFO 7483 mycelium | [15] |
| | | | | | *Mucor* sp. JX23 growing cell culture/mycelium | [37] |
| | | | | | *Actinidia chinensis*[a),b),c)], *Daucus carota*[a),c)], *Helianthus annuus*[a),b),c)], *Nicotiana tabacum*[a),b),c)], *Polygonum persicaria*[a),b),c)], *Rauwolfia manii*[a)] *Solanum melanogena*[b),c)], and *Tagetes patula*[a),c)] | [43] |
| | | | | | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| | | | | | *Actinomyces* sp.[d)] | [62] |
| | | | | | *Pyrococcus furiosus* DSM 3638 growing cells | [70,82] |
| | | | | | Purified Mo-AOR from *Clostridium formicoaceticum* DSM 92[g)] | [60] |
| | | | | | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[e)] | [59] |
| 2 | H | H | Cl | H | *Trametes versicolor* IFO 4941 mycelium | [15] |
| 3 | OMe | H | H | H | *Aspergillus flavus* DSM 1959 mycelium | [15] |
| 4 | H | OMe | OMe | H | *Trametes* sp. mycelium | [16] |
| | | | | | *Trametes hirsutus* IFO 4917 mycelium | [15] |
| | | | | | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 5 | H | $OCH_2O$ | | H | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 6 | H | OMe | H | OMe | *Aspergillus niger* ATCC 4192 mycelium | [15] |
| 7 | H | OMe | OMe | OMe | *Trametes* sp. mycelium | [16] |
| | | | | | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 8 | H | OMe | OH | H | *Trametes* sp. mycelium | [16] |
| | | | | | *Mycobacterium phlei* DSM 43286 and *Psilocybe zapotekorum* DSM 1891 mycelium | [15] |
| | | | | | *Sporotrichum pulverulentum* ATCC 3262917 mycelium | [18] |
| | | | | | *Fomes fomentarius* mycelium | [17] |
| | | | | | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 recombinant CAR heterologously expressed in *E. coli* and purified from it [f)] and resting *E. coli* cells overexpressing *Ni*CAR | [47,50] |
| 9 | H | OMe | OH | OMe | *Trametes* sp. mycelium | [16] |
| 10 | H | H | OMe | H | *Trametes* sp. mycelium | [16] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[f)] | [46] |
| 11 | OH | H | H | H | *Corynespora cassiicola* CBS 12925 mycelium | [15] |
| | | | | | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 12 | H | OH | H | H | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 13 | H | OH | OH | H | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 14 | H | OH | OMe | H | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |
| 15 | H | H | OH | H | *Trametes* sp. mycelium | [16] |
| | | | | | *Fomes fomentarius* mycelium | [17] |

| | | |
|---|---|---|
| | Purified CAR from *Neurospora crassa* SY7A[f)] | [44] |

[a)] Resting cells from 7-day-old submerged cultures resuspended in buffer; [b)] Resting cells from 7-day-old submerged cultures, lyophilized and resuspended in buffer; [c)] Resting cells from 7-day-old submerged cultures, lyophilized and resuspended in biphasic system; [d)] Lyophilized and permeabilized whole cells; [e)] In the presence of $CAV^{+\bullet}$; [f)] In the presence of ATP and NADPH; [g)] In the presence of $TMV^{+\bullet}$

Soon after the very first report [13], carboxylate reducing activity has also been found in other fungi - in members of the Ascomycota and Zygomycota families. Bachmann et al. reported *in vivo* reduction of salicylic acid to salicyl alcohol by *Neurospora crassa* [33]. Similarly to the activities found in *Hebeloma sacchariolens* [32], mycelium from *Aspergillus niger* Perlman Wisconsin 72-4 was reported to convert benzoic acid as well as 2- and 4-aminobenzoic acid to the respective aldehydes [34]. Under anaerobic conditions, benzaldehyde was reduced further [34], similarly to other Aspergilli, which converted the acid substrates to the alcohols. Also resting cells of *Hormoconis resinae* F328 (*Amorphotheca resinae*, fungus from the division Ascomycota) reduced benzoates to the alcohols [35,36]. Mycelium of *A. niger* ATCC 9142, e.g., acted on 3-methoxybenzoic and 3,5-dimethoxycinnamic acid, and *A. flavus* DSM 1959 reduced 2-methoxycinnamic acid, but only to a very small extent (1.6%). Also *Corynespora cassiicola (melonis)* IFO 7483 was reported to reduce 3-methoxybenzoic, 4-anisic, and also cinnamic acid to the respective alcohols, whereas *C. cassiicola* CBS 12925 only reduced the 2-hydroxy derivative [15]. More recently, also *Mucor* sp. JX23 (division Zygomycota) was shown to reduce cinnamic acid to cinnamic alcohol (Table 2) [37]. *Glomerella cingulata* (the so called *Malus* strain, because it had been isolated from apples) and *Gloeosporium olivarum* were able to reduce a number of racemic 2-alkyl-2-aryloxyacetic acids to the respective alcohols (Table 5, entry 48) [38,39]. Notably, the authors observed a kinetic resolution to (*S*) alcohols and (*R*) acids which is, to our knowledge, the first report of enantioselective enzymatic carboxylate reduction [39]. In a following study, these results were confirmed with further examples in which *Glomerella cingulata* (*Prunus* strain), *Gloeosporium olivarum* and *Gloeosporium laeticolor* reduced 2-phenyl-, 2-benzyloxy- and 2-(2-furfuryl)propionic acid [40]. *Botrytis cinerea* (four strains: 5889/4, 5901/2, 5882/1, and 5909/1) was the first fungus to be used for the conversion of purely aliphatic substrates (*trans*-3-hexenoic acid and *trans*-3-octenoic acid, Table 5, entries: 25 and 30) to the corresponding alcohols [41].

**Table 3. Phenylacetic acid derivatives as substrates for carboxylate reducing activities**




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | Biocatalyst | Ref |
|---|---|---|---|---|---|---|
| 1 | H | H | H | H | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | | | | *Clostridium thermoaceticum* DSM 521resting cells[b)] | [59] |
| | | | | | *Clostridium formicoaceticum* DSM 92 crude extract[c)] | [81] |
| 2 | OMe | H | H | H | *Trametes hirsutus* IFO 4917 mycelium | [15] |
| 3 | H | H | H | OMe | *Clostridium thermoaceticum* DSM 521resting cells[b)] | [59] |
| 4 | H | OH | OH | H | *E. coli* resting cells heterogously expressing recombinant *NiCAR* from *Nocardia iowensis* sp. NRRL 5646 | [52] |
| 5 | H | H | H | OH | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | $NO_2$ | H | H | H | *Trametes versicolor* IFO 4941 mycelium | [15] |
| 7 | H | $NO_2$ | H | H | *Trametes versicolor* IFO 4941 mycelium | [15] |
| 8 | H | H | H | Me | *Glomerella cingulata* (*Prunus* and *Malus* strains), *Gloeosporium olivarum* and *Gloeosporium laeticolor* growing cell culture | [39,40] |
| | | | | | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | | | | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | | | | *Clostridium thermoaceticum* DSM 521resting cells[b)] | [59] |
| | | | | | *Clostridium formicoaceticum* DSM 92 crude extract[c)] | [81] |
| | | | | | *Clostridium thermoaceticum* resting cells[b)] | [55] |
| 9 | H | H | H | COOH | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 10 | H | H | H | $CH_2COOH$ | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 11 | H | H | *i*Bu | Me (*R*) | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |

[a)] In the presence of ATP and NADPH; [b)] In the presence of CO and MV; [c)] In the presence of CO

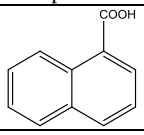
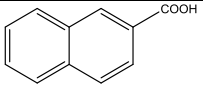
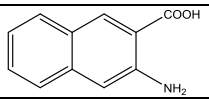
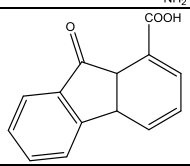
## 3 Aerobic carboxylate reductions by bacteria

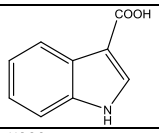
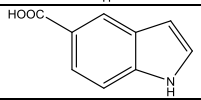
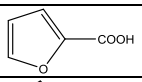
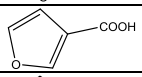
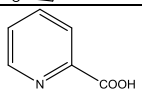
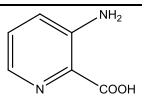
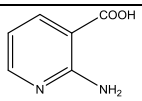
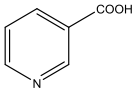
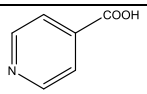
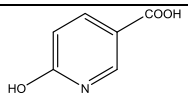
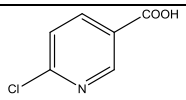
*Mycobacterium phlei* DSM 43286 (an acid-fast bacterium) reduced ferulic acid both to aldehyde and alcohol. Interestingly, this species was discovered in the course of a broad screening approach in which diverse species were tested. In this case, more aldehyde (13.1%) than alcohol was found. This indicates that the internal alcohol dehydrogenase(s) are not as active like e.g. in *Psilocybe zapotekorum* DSM 1891, belonging to the division Basidiomycota [15]. Furthermore also resting cells of the gram-positive bacterium *Nocardia asteroides* JCM3016 reduced benzoates to the corresponding alcohols [35]. Vanillic acid was reduced to the vanillyl alcohol with another subspecies of *Nocardia: Nocardia iowensis* strain NRRL 5646 [42].

## 4 Aerobic carboxylate reductions by plants

Recently, lyophilized plant cell cultures were screened for their ability to reduce carboxylic acids and several plants e.g. *Nicotiana tabacum* and *Helianthus annuus* were able to reduce cinnamic, hexanoic and octanoic acid to the corresponding aldehydes (in case of three species) and alcohols in reduction yields from 2 to 80% (w/w) (Table 2, entry 1 and Table 5, entries: 21 and 29) [43].

**Table 4. Poly- and heterocyclic carboxylic acids as substrates for carboxylic acid reducing activities**

| | Compound structure | Biocatalyst | Ref |
|---|---|---|---|
| 1 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 2 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | *Trametes versicolor* mycelium | [13] |
| 3 |  | *Hebeloma sacchariolens* fruit bodies and homogenate | [32] |
| 4 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |

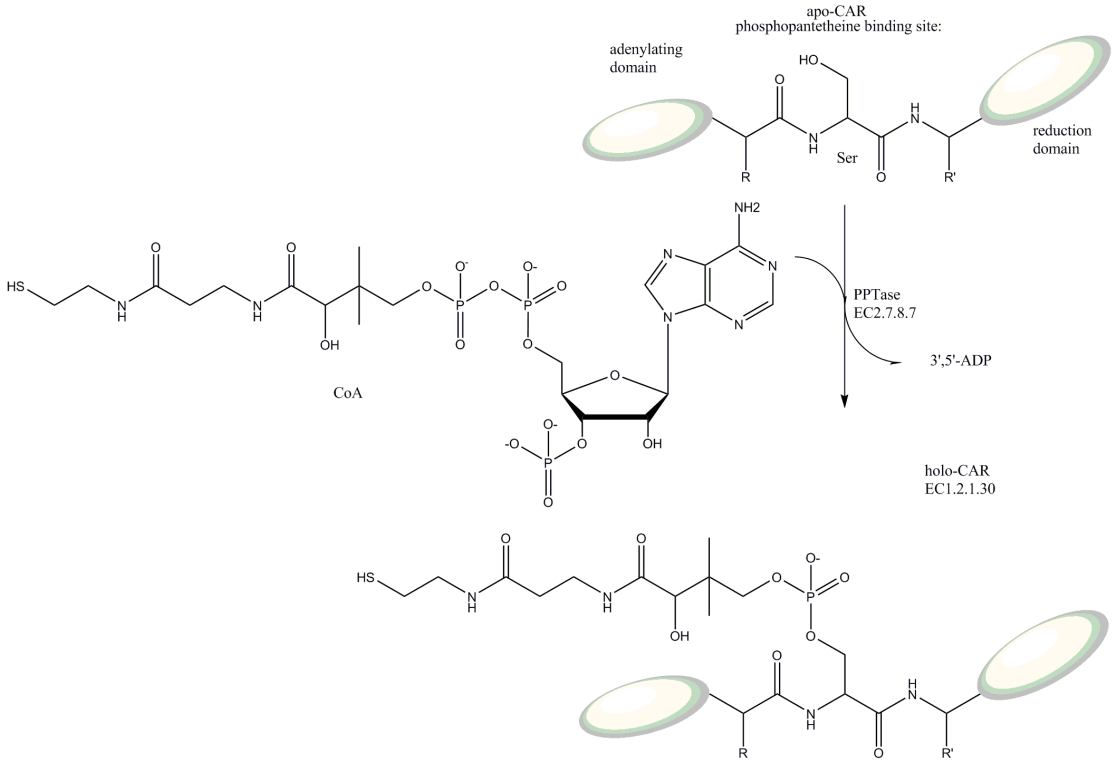
| | | | |
|---|---|---|---|
| 5 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 6 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 7 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 8 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| 9 |  | Partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[a)] | [59] |
| 10 |  | *Hebeloma sacchariolens* fruit bodies and homogenate | [32] |
| 11 |  | *Hebeloma sacchariolens* fruit bodies and homogenate | [32] |
| 12 |  | *Nocardia iowensis* sp. NRRL 5646 crude extract[a)] | [46] |
| | | Partially purified W-AOR from *Clostridium thermoaceticum*[b)] | [59] |
| 13 |  | *Clostridium thermoaceticum* DSM 521resting cells[c)] and partially purified W-AOR from *Clostridium thermoaceticum* DSM 521[b)] | [59] |
| 14 |  | *Clostridium thermoaceticum* DSM 521resting cells[c)] | [59] |
| 15 |  | *Clostridium thermoaceticum* DSM 521resting cells[c)] | [59] |
| | | Purified Mo-AOR from *Clostridium formicoaceticum* DSM 92 in the presence of $TMV^{+\bullet}$ | [60] |

[a)] In the presence of ATP and NADPH; [b)] In the presence of $CAV^{+\bullet}$; [c)] In the presence of CO and MV

## 5 Enzyme discovery and carboxylate reductions with purified and overexpressed enzymes

In contrast to the biotransformations using organisms, parts of organisms or cell extracts described in the previous sections, a range of aryl-aldehydes were prepared with a purified enzyme from *Neurospora crassa* – an aryl-aldehyde:NADP-oxidoreductase. This enzyme, isolated by Gross et al., was active for the reduction of cinnamic acid and its derivatives (Table 2). A first exploration of the reaction mechanism revealed that the enzyme was NADP(H) and ATP dependent and a positive influence of $Mg^{2+}$ and DTT on the enzyme's activity was found [44,45]. These results were confirmed by the group of Rosazza and co-workers in 1997 with a homologous enzyme isolated from *Nocardia iowensis* strain NRRL 5646, a gram-positive bacterium. They purified and characterized a carboxylate reductase from *Nocardia* sp. NRRL 5646 (*Ni*CAR) and investigated 58 substrates, mostly benzoic acid

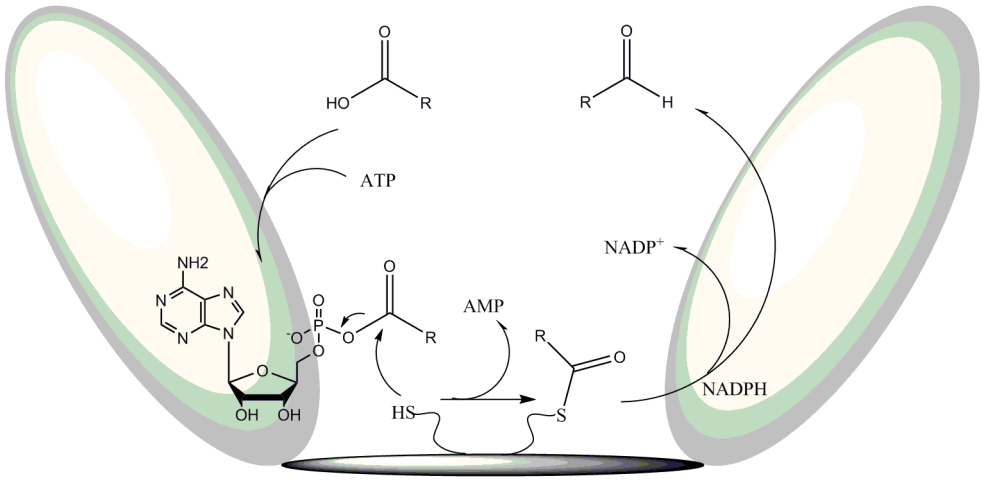
and its halogen, methoxy, acetyl, nitrile, phenyl, phenoxy and benzoyloxy derivatives as well as toluic, anisic (Table 1), naphthoic, furoic, nicotinic (Table 4), phenylmalonic, -succinic, -acetic (Table 5) and 2-phenylpropionic acid (Table 3). From all substrates, 3-chlorobenzoic acid was superior to 3-bromobenzoic, 4-chlorobenzoic and benzoic acid. In general, 3-substituted benzoic acids were converted best, whereas substitutions in 2-position resulted in poor conversions. For all variants of nitrobenzoic acids, *Ni*CAR did not show any activity. Due to this substrate scope, the enzyme was classified as aryl-aldehyde dehydrogenase ($NADP^+$) with the E.C. number 1.2.1.30. An investigation with ibuprofen as a substrate showed that the enzyme was (*R*)-selective [46]. In 2000, Rosazza and co-workers found that vanillic acid was reduced to vanillyl alcohol with *Nocardia* resting cells, whereas with the purified enzyme the reduction evidently stopped at the stage of the aldehyde vanillin: an indication that *Nocardia* cells have to have one or more dehydrogenases responsible for further reduction of vanillin to vanillyl alcohol [42]. In 2004, the gene coding for the *Ni*CAR protein was discovered and cloned for heterologous expression in *E. coli* [47], however, a functional protein was only obtained after a post-translational phosphopantetheinylation. Specifically, the 4-phosphopantetheine moiety of CoA is covalently attached to a conserved serine residue of the CAR apoprotein and thereby it is converted into the active holoprotein (Scheme 3) [48]. The reaction is catalyzed by a phosphopantetheinetransferase (PPTase), the role of which was previously documented in non-ribosomal peptide and fatty acid synthesis [49].




**Scheme 3. Transfer of a phosphopantetheine moiety from the donor (CoA) to conserved residue of apo-CAR catalyzed by PPTase. PPT-binding site motif: LGG-x-S-xx-A.** (Adapted from Ref. [48])

Venkitasubramanian et al. elucidated the catalytic function of the carboxylate reductase enzyme, and discovered that 3 domains are necessary for catalysis and suggested a reaction mechanism (Scheme 4) [48]:

- adenylating domain to activate the free, deprotonated carboxylic acid as an adenosyl phosphate at the expense of ATP,
- phosphopantethein binding site (which needs the additional PPTase to attach phosphopantethein at the expense of CoA to the enzyme, Scheme 3) that attacks the carbonyl of the activated ester to form a thioester,
- reduction domain that eventually reduces the thioester and releases an aldehyde.




**Scheme 4**. **Proposed catalytic cycle of holo-CAR reduction of benzoic acid to benzaldehyde**. (Adapted from Ref. [48])

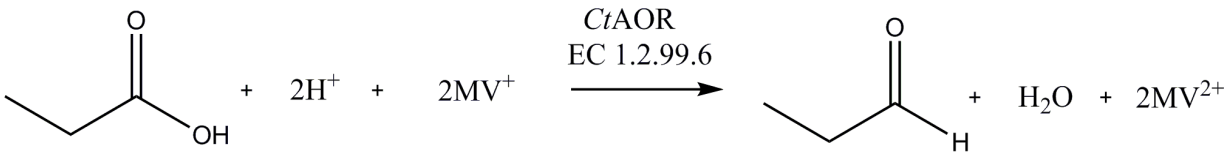
Combining mechanistic and sequence information of the *Ni*CAR enzyme with previous results of vanillic acid reduction, *Ni*CAR was expressed in *E. coli*. In addition to the *Ni*CAR sequence, the plasmid carried also the *Bacillus subtilis* PPTase and glucose dehydrogenase for efficient cofactor recycling [50] to improve vanillic acid reduction further [42]. Alternatively, Hansen et al. used *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to generate microbial strains for the production of vanillin. The required phosphopanteteinylation was achieved by coexpression of PPTase from *Corynebacterium glutamicum*. The overreduction to vanillyl alcohol by host enzymes was overcome by the knockout of the host alcohol dehydrogenase *ADH6* [51]. In this study an obstacle, the over-reduction was an unexpected lucky chance in another application of the *Ni*CAR enzyme: 3,4-dihydroxyphenylacetic acid was reduced to the antioxidant 3-hydroxytyrosol by resting *E.coli* cells co-expressing *Ni*CAR and the PPTase from *E. coli* (Table 3, entry 4) [52].

The substrate scope of the *Ni*CAR enzyme was further enlightened with a purified enzyme preparation. Several non-aromatic carboxylic mono and diacids were tested as substrates making use of the photometric detection of NADPH consumption. Although 2-ketoglutaric acid appeared to be the best substrate [50] it needs to be stressed that a comparison of mono- and diacids in a cofactor based activity assay is difficult, because no

information is available whether only one or both acid functionalities are being reduced. With respect to aliphatic compounds, *Ni*CAR was also considered as a catalyst for fatty acid reduction [53], however, a homologous protein from *Mycobacterium marinum* (*Mm*CAR), sharing 62% identity with *Ni*CAR proved capable of reducing $C_6$-$C_{18}$ fatty acids to the respective aldehydes. With this carboxylate reduction as the key step, cell factories expressing a pathway of four exogenous enzymes (e.g. the aldehyde reductase from *Synechocystis* sp. PCC 6803) were developed for the preparation of fatty alcohols [54].

## 6 Anaerobic carboxylate reductions by bacteria

In addition to carboxylate reductase activities described in the previous chapters, microbial reductions of carboxylic acids under anaerobic conditions have been reported. To our knowledge, the first publication concerning this type of reaction appeared in 1987. In presence of CO, $H_2$ or formate, methyl viologen (MV) and whole cells of *Clostridium thermoaceticum*, a range of aliphatic and aromatic carboxylic acids were reduced to the respective alcohols (Scheme 5), among which hexanedioic acid appeared to be the best substrate. It was reasoned that if the redox potential of carbon monooxidase from *C. thermoaceticum* had been quoted for -560 mV, whereas the redox potential of carboxylate/aldehyde had been estimated for -550 mV, this microbe should be able to reduce carboxylic acids. However, the addition of methyl viologen as a redox mediator proved to be essential to promote this reaction [55]. Crude extract or supernatant of broken cells of *C. formicoaceticum* DSM 92 was reported to reduce branched and unbranched, saturated and unsaturated carboxylates at the expenses of CO to the corresponding alcohols. Interestingly, in contrast to *C. thermoaceticum* DSM 521, it was found that the reduction of carboxylates with *C. formicoaceticum* DSM 92 proceeded faster in the absence of violegens [56]. The enzymes responsible for carboxylic acid reduction in these two organisms were isolated and characterized to be highly oxygen sensitive tungsten enzymes (W-AOR, carboxylate reductases, E.C.1.2.99.6) [57–59], however, also a less oxygen sensitive molybdenum containing aldehyde oxidoreductase (Mo-AOR) was identified [60]. Similar to the *Ni*CAR enzyme, both purified *Cf*AOR reduced carboxylic acids selectively to aldehydes, despite the apparent differences of their catalytic mechanism, whereas whole cell bioconversions catalyzed by *Clostridia* typically yield alcohols [55].




**Scheme 5. Carboxylic acid reduction catalyzed by tungsten enzyme E.C.1.2.99.6. Non-activated acid is reduced by reduced viologens to aldehydes**. (Adapted from Ref. 59).

Perez et al. reported the use of syngas in combination with whole cells of *Clostridium ljungdahlii* and *Clostridium ragsdalei* for reducing additional aliphatic carboxylic acids such as acetic, propionic, *n*-butyric, *n*-valeric and *n*-caproic acid to the corresponding alcohols (Table 5, entries: 1, 2, 6, 15 and 21). Syngas is a synthetic mixture of CO, $CO_2$ and $H_2$,

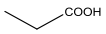
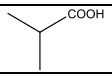
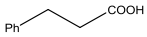
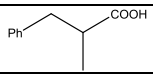
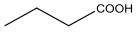
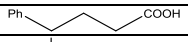
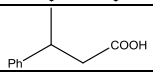
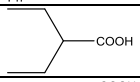
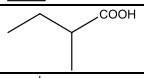
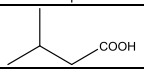
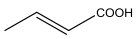
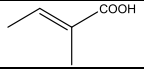
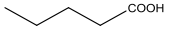
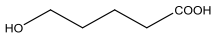
generated during thermal pyrolysis of lignocelluloses. Hence, the idea was to apply carboxydotrophic bacteria that consume easily available syngas and produce valuable biofuel such as *n*-butanol [61].

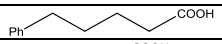
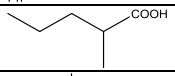
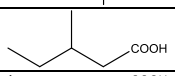
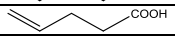
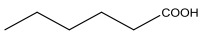
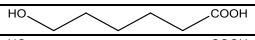
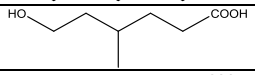
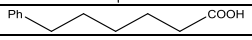
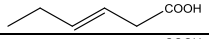
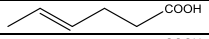
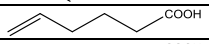
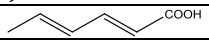
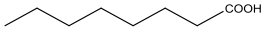
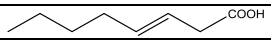
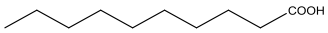
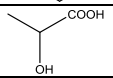
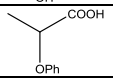
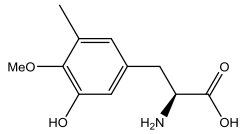
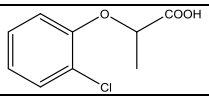
Aromatic carboxylic acids were reported as substrates of another anaerobic bacteria species, namely *Actinomyces* sp. The reaction proceeded at anaerobic conditions at 50 °C and gave the corresponding aldehydes in yields from 5 to 67%. Since the aldehyde products were detected via their 2,4-dinitrophenylhydrazones, further reduction to alcohol may not be excluded. The *Actinomyces* sp. cells were lyophilized and additionally permeabilized with toluene in order to allow substrate access. Derivatives of acrylic acid such as cinnamic and crotonic acid were converted sluggishly in comparison to the best substrates vanillic acid and 3,4,5-trimethoxybenzoic acid [62].

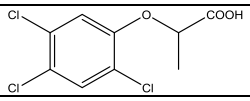
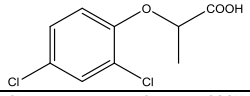
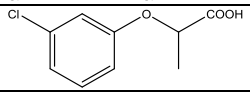
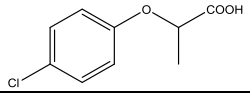
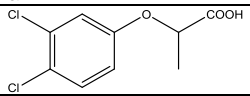
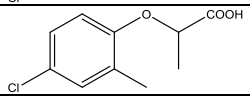
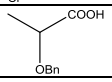
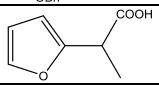
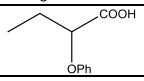
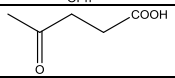
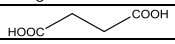
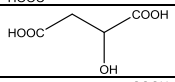
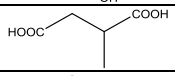
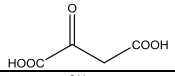
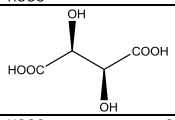
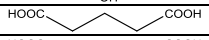
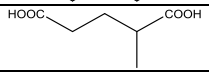
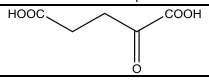
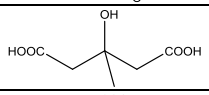
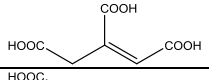
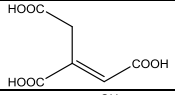
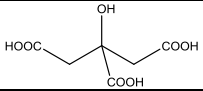
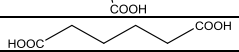
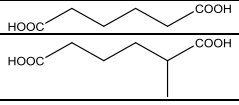
The Desulfovibrionales species also exhibits carboxylic acid reducing activity under anaerobic conditions. A culture of *Desulfomicrobium escambiense* reduced 3-chloro and 3-bromobenzoate to the corresponding benzyl alcohols in the presence of pyruvate [63]. Interestingly, these compounds were not converted by *Desulfovibrio vulgaris* PY1, which reduced nine other halogenated benzoic acids under the same conditions. However, when MV, FAD, FMN or ferredoxin as electron carrier and pyruvate or $H_2$ as electron donor were supplied to cell free extracts of this organism, 3-chlorobenzoate could be reduced as well. Also reduction of the sulfate content of the medium allowed this conversion [64]. To our knowledge, these two enzymes were not isolated or characterized in detail and they are not available in recombinant form to date.

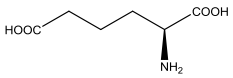
Mixed anaerobic cultures enjoy increasing interest, because of their potential use for the conversion of waste materials to bioproducts, especially within so called "carboxylate platform". This term refers to carboxylates generated with undefined mixed cultures from organic feedstocks derived from industrial and agricultures wastes. During primary fermentation, acetate, propionate, lactate and *n*-butyrate are generated and subsequently proceeded (*inter alia* reduced to the alcohols) during secondary fermentation [65]. Technical and economic analysis of such processes as well as the aforementioned syngas fermentation showed that both methods are worth to be commercialized [66]. Such mixed cultures were e.g. used for the production of ethanol, propanol and *n*-butanol from acetate, propionate and *n*-butyrate, respectively. Specifically, granular sludge from up-flow anaerobic sludge blanket reactors was used for this purpose. All three carboxylic acids, as well as the hydrogen needed as an electron donor, were derived from fermentative waste biomass acidification and the respective alcohols represented about 50% of the obtained products, whereas the by-products were dominated by methane (30%) [67]. Alternatively, hydrogen was replaced with an electrode as electron donor and $MV^+$ was used to accelerate acetate reduction [68]. In a similar approach, Sharma et al. recently used a mixed culture of sulfate-reducing bacteria for bio-electrocatalyzed reduction of acetic and butyric acid via direct electron transfer. The products of this reduction were mainly ethanol and butanol, although also caproate and acetone were detected [69].

**Table 5. Aliphatic carboxylic acids and derivatives thereof**

| | Compound structure | Biocatalyst | Ref |
|---|---|---|---|
| 1 |  | *Clostridium thermoaceticum* resting cells[a)] | [55] |
| | | *Clostridium formicoaceticum* DSM 92 crude extract[b)]<br>Purified W-AOR and Mo-AOR from *Clostridium formicoaceticum* DSM 92[c)] | [81]<br>[57,60] |
| | | *Clostridium ljungdhalii* ERI-2 (ATTC 55380) and *Clostridium ragsdalei* P11 (ATTC BAA-622)[d)] | [61] |
| | | Mixed culture from anaerobic granular sludge | [67,68] |
| | | Mixed culture of anaerobic sulfate reducing bacteria | [69] |
| | | *Thermococcus* sp.1 ES-1 in the presence of $MV^+$ | [74] |
| 2 |  | *Clostridium thermoaceticum* DSM 92 resting cells[a)]<br>Partially purified W-AOR from *Clostridium thermoaceticum* DSM 92[e)] or in the presence $MV^+$ | [55]<br>[34,61] |
| | | *Clostridium formicoaceticum* crude extract[b)]<br>Purified W-AOR and Mo-AOR from *Clostridium formicoaceticum*[c)] | [81]<br>[57,60] |
| | | *Clostridium ljungdhalii* ERI-2 (ATTC 55380) and *Clostridium ragsdalei* P11 (ATTC BAA-622)[d)] | [61] |
| | | Mixed culture from anaerobic granular sludge | [67] |
| | | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| 3 |  | *Clostridium ljungdhalii* ERI-2 (ATTC 55380) and *Clostridium ragsdalei* P11 (ATTC BAA-622)[d)] | [61] |
| 4 |  | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| | | *Pyrococcus furiosus* DSM 3638 growing and resting cells | [70,71,82] |
| 5 |  | *Clostridium thermoaceticum* resting cells[a)] | [55] |
| 6 |  | *Clostridium thermoaceticum* resting cells[a)] | [55,59] |
| | | *Clostridium formicoaceticum* crude extract[b)]<br>Purified Mo-AOR from *Clostridium formicoaceticum* DSM92[c)] | [81]<br>[60] |
| | | *Clostridium ljungdhalii* ERI-2 (ATTC 55380) and *Clostridium ragsdalei* P11 (ATTC BAA-622)[d)] | [61] |
| | | Mixed culture from anaerobic granular sludge | [67] |
| | | Mixed culture of anaerobic sulfate reducing bacteria | [69] |
| | | *Pyrococcus furiosus* DSM 3638 growing and resting cells | [70,71,82] |
| 7 |  | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| 8 |  | *Clostridium thermoaceticum* resting cells[a)] | [55] |
| 9 |  | *Pyrococcus furiosus* DSM 3638 resting cells | [71] |
| 10 |  | *Clostridium thermoaceticum* DSM 521resting cells[a)] | [59] |
| 11 |  | *Clostridium thermoaceticum* DSM 521resting cells[a)] | [55,59] |
| 12 |  | *Actinomyces* sp.[f)] | [62] |
| | | Purified Mo-AOR from *Clostridium formicoaceticum* DSM 92[c)] | [60] |
| | | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| 13 |  | *Clostridium thermoaceticum* resting[a)] | [55] |
| 14 |  | *Clostridium thermoaceticum* resting cells[a)] | [55] |
| | | *Clostridium formicoaceticum* DSM 92 crude extract[b)] | [81] |
| 15 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| | | *Clostridium formicoaceticum* DSM 92 crude extract[b)] | [81] |
| | | *Clostridium ljungdhalii* ERI-2 (ATTC 55380) *and Clostridium ragsdalei* P11 (ATTC BAA-622)[d)] | [61] |
| | | *Pyrococcus furiosus* DSM 3638 resting cells | [71] |
| 16 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |

| | | | |
|---|---|---|---|
| 17 |  | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| 18 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 19 |  | *Clostridium thermoaceticum* DSM 521resting cells[a)] | [59] |
| 20 |  | *Pyrococcus furiosus* DSM 3638 resting cells | [71] |
| 21 |  | *Clostridium formicoaceticum* resting cells[b)] | [81] |
| | | *Actinidia chinensis*[g)], *Daucus carota*[g),i)], *Helianthus annuus*[g),h),i)], *Nicotiana tabacum*[g),h),i)], *Phytolacca decandra*[g)], *Polygonum persicaria*[g)], *Rauwolfia manii*[g)], *Solanum melanogena*[g),h),i)], and *Tagetes patula*[i)] | [43] |
| | | *Clostridium ljungdhalii* ERI-2 (ATTC 55380) and *Clostridium ragsdalei* P11 (ATTC BAA-622)[d)] | [61] |
| | | *Pyrococcus furiosus* DSM 3638 growing and resting cells | [70,71,82] |
| 22 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 23 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 24 |  | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| 25 |  | *Botrytis cinerea* 5889/4, 5901/2, 5882/1, and 5909/1 mycelium | [41] |
| 26 |  | *Pyrococcus furiosus* DSM 3638 resting cells | [71] |
| 27 |  | *Pyrococcus furiosus* DSM 3638 resting cells | [71] |
| 28 |  | *Clostridium thermoaceticum* resting cells[a)] | [55] |
| 29 |  | *Clostridium formicoaceticum* DSM 92 crude extract[b)] | [81] |
| | | Purified Mo-AOR from *Clostridium formicoaceticum* DSM 92[c)] | [60] |
| | | *Actinidia chinensis*, *Daucus carota*, *Helianthus annuus*, *Phytolacca decandra*, *Polygonum persicaria*, *Rauwolfia manii*, *Solanum melanogena*, and *Tagetes patula*[g)] | [43] |
| 30 |  | *Botrytis cinerea* 5889/4, 5901/2, 5882/1, and 5909/1 mycelium | [41] |
| 31 |  | *Clostridium formicoaceticum* DSM 92 crude extract[b)] | [81] |
| | | *Pyrococcus furiosus* DSM 3638 growing and resting cells | [70,71] |
| 32 | Fatty acids $C_6$-$C_{18}$ | CAR from *Mycobacterium marinum* overexpressed in *E. coli* | [54] |
| 33 | Fatty acids $C_{12}$-$C_{18}$ (saturated and unsaturated) | Growing cells of *E. coli* expressing fatty acyl-ACP reductase from *Synechococcus elongatus* | [76] |
| | | Growing cells of *E. coli* expressing fatty acyl-CoA reductase from *Arabidopsis thaliana* | [83] |
| 34 | Fatty acids $C_{14}$-$C_{22}$ (saturated) and Fatty acids $C_{16}$-$C_{20}$ (unsaturated) | Growing cells of *S. cerevisiae* expressing fatty acyl-CoA reductase from *Apis mellifera* | [84] |
| 35 | Myristic acid ($C_{14}$) | *Photobacterium phosphoreum* transferase, synthetase and reductase | [75] |
| | | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| | | Growing cells of *S. cerevisiae* expressing fatty acyl-CoA reductase from *Euglena gracilis* | [85] |
| 36 | Palmitic acid ($C_{16}$) | *Pyrococcus furiosus* DSM 3638 growing cells | [70] |
| | | Growing cells of *S. cerevisiae* expressing fatty acyl-CoA reductase from *Euglena gracilis* | [85] |
| 37 |  | *Nocardia asteroides* JCM 3016 resting cells | [35,36] |
| 38 |  | *Nocardia iowensis* sp. NRRL 5646[h)] | [50] |
| | | *Clostridium thermoaceticum* resting cells[a)] | [55] |
| 39 |  | *Glomerella cingulata (Prunus* and *Malus* strain), *Gloeosporium olivarum* and *Gloeosporium laeticolor* growing cell culture | [38–40] |
| 40 |  | *Streptomyces lavendulae* SfmC, purified and treated with PPTase[j)] | [80] |
| 41 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |

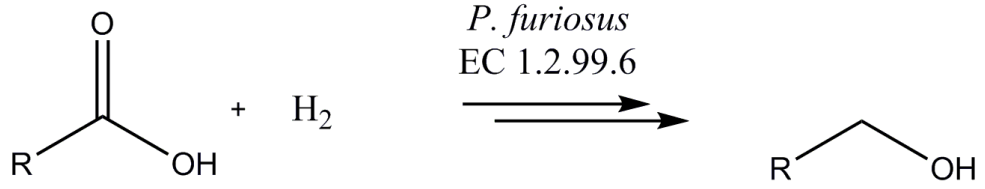
| | | | |
|---|---|---|---|
| 41 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| | | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 42 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| | | *Clostridium thermoaceticum* resting cells[a)] | [59] |
| 43 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| 44 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| | | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 45 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| 46 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| 47 |  | *Glomerella cingulata (Prunus* and *Malus* strain), *Gloeosporium olivarum* and *Gloeosporium laeticolor* growing cell culture | [40] |
| 48 |  | *Glomerella cingulata (Prunus* and *Malus* strain), *Gloeosporium olivarum* and *Gloeosporium laeticolor* growing cell culture | [40] |
| 49 |  | *Gloeosporium olivarum* growing cell culture | [39] |
| 50 |  | *Pyrococcus furiosus* DSM 3638 resting cells | [71] |
| 51 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [55,59] |
| 52 |  | *Nocardia iowensis* sp. NRRL 5646[j)] | [50] |
| 53 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 54 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 55 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 56 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [55,59] |
| 57 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |
| 58 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 59 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 60 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 61 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 62 |  | *Nocardia iowensis* sp. NRRL 5646[j),k)] | [50] |
| 63 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [55,59] |
| 64 |  | *Clostridium thermoaceticum* DSM 521 resting cells[a)] | [59] |

| | | | |
|---|---|---|---|
| 65 |  | *Schizosaccharomyces pombe* wt 972, cell free extract, *Schizosaccharomyces pombe* Lys1p expressed in *E. coli* and *Candida albicans* Lys2p expressed in *E. coli* | [77,78] |

[a] In the presence of CO and MV; [b] In the presence of CO; [c] In the presence of $TMV^{+\bullet}$; [d] Growing cells with syngas; [e] In the presence of $CAV^{+\bullet}$; [f] Lyophilized and permeabilized whole cells; [g] Resting cells from 7-day-old submerged cultures resuspended in buffer; [h] Resting cells from 7-day-old submerged cultures, lyophilized and resuspended in buffer; [I] Resting cells from 7-day-old submerged cultures, lyophilized an resuspended in biphasic system; [j] In the presence of ATP and NADPH; [k] Recombinant CAR heterogously exspressed in *E. coli* and purified from it;

## 7 Anaerobic carboxylate reductions by Archea

Also hyperthermophilic archea such as *Pyrococcus furiosus* DSM 3638 exhibit reductive activities towards carboxylic acid substrates. It was shown that *P. furiosus* batch cultures at anaerobic conditions and 90°C reduced *trans*-cinnamic acid and 3-phenylpropionic acid to the corresponding alcohols in 67 and 69% conversion, respectively. Among aliphatic acids, the best accepted substrate was hexanoic acid with a reduction yield of 38% to hexanol [70]. In another study, Ni et al. also used *P. furiosus* as the hydrogenating biocatalyst, which can combine $H_2$ oxidation (catalyzed by a hydrogenase) with carboxylic acid reduction (catalyzed by an aldehyde ferredoxin reductase – AOR), (Scheme 6). Eleven carboxylic acids were reduced to alcohols in up to 99% conversion. Medium-chain aliphatic acids were reduced within 24h whereas the reaction rate for longer chain aliphatic acids was slower. Hydrogen ($H_2$), that is oxidized by a hydrogenase enzyme in this organism, serves as reducing equivalent and the carboxylate reduction itself is catalyzed by an oxygen sensitive tungsten pterin enzyme [71]. A particular challenge in the reduction of carboxylic acids and their derivatives is the selective termination of the reduction process at the stage of the aldehyde. In *P. furiosus* catalyzed bioreductions, simple medium engineering led to the success: specifically, the aqueous reaction medium was replaced by hexadecane. This substitution allowed for aldehyde accumulation, although the overall biohydrogenation activity decreased [71]. The enzyme catalyzing the above mentioned carboxylic acid reduction was isolated already two decades before by Mukund and Adams and initially described as red-colored tungsten-containing protein (RTP) [72]. However, further investigation showed that RTP is in fact an inactive form of an aldehyde ferredoxin reductase (AOR), which, in contrast to RTP, contains a catalytically essential W-SH group [73]. Another tungsten-containing AOR, also from a hyperthermophilic archaeon - *Thermococcus* ES-1 - showed high homology to *P. furiosus* AOR and *C. formicoaceticum* AOR and the enzyme itself was able to reduce acetate at 85°C with methyl viologen ($MV^+$) as the electron donor, although the observed activity was much lower than the reverse aldehyde oxidation [74].

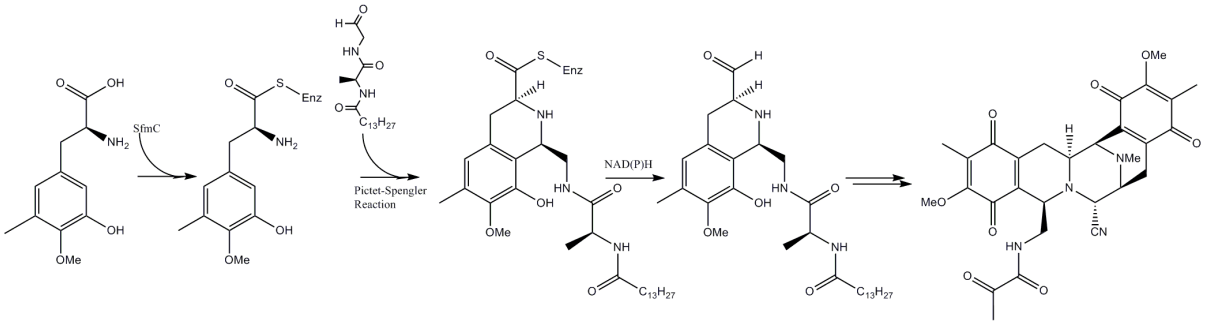



**Scheme 6.** ***Pyrococcus furiosus*** **catalyzed hydrogenation of carboxylic acids**

## 8 Other examples of carboxylate reducing enzymatic activities

Biocatalytic reduction of carboxylic acids is also well known from bacterial bioluminescence, where the long chain aldehydes that are required for the luminescent reaction are generated by fatty acid reduction ([75], and references therein) and fatty acid biosynthesis ([76], and references therein). Also in one of the biosynthetic pathways of lysine in fungi, the substrate α-aminoadipic acid is reduced to α-aminoadipate-δ-semialdehyde by α-aminoadipate reductase (E.C.1.2.1.31) [77,78]. This enzyme class appears to be highly substrate specific, as no other substrate was reported to date.

An enzyme possessing carboxylic acid reduction ability has been found in the course of antibiotic biosynthesis in *Streptomyces griseus*. The protein products of griC and griD catalyze the ATP and NADPH dependent reduction of 3-amino-4-hydroxybenzoic acid to its aldehyde which serves as the precursor of grixazone A (one of two ingredients of a yellow pigment produced by *Streptomyces griseus*) [79]. In a similar report, the non-ribosomal polypeptide synthetase SfmC from *Streptomyces lavendulae* catalyzed the reduction of the carboxylate from a tyrosine derivative (Table 5, entry 40), to its aldehyde as the key step to the antitumor antibiotic Saframycin A (Scheme 7) [80].




**Scheme 7. Carboxylate reduction in Saframycin biosynthesis.** (Adapted from Ref. [80])

Summarizing, three enzyme classes catalyze the reduction of free carboxylic acids to aldehydes. E.C. 1.2.99.6 consists of oxygen sensitive metal dependent proteins that were not extensively studied or used for biocatalysis so far. E.C. 1.2.1.30 and 31 are proteins, consisting of $NADP^+$ dependent aryl-aldehyde dehydrogenase and α-aminoadipate reductase. In addition, non-ribosomal peptide synthetases may catalyze carboxylate reductions. From fatty acid biosynthesis and bioluminescence, fatty acyl-ACP reductases and fatty acyl-CoA reductases are known, which formally also reduce acids, however, bound to high molecular weight residues (Table 6). Therefore, these latter enzymes have not been reviewed in detail here.

**Table 6. Enzyme classes catalyzing carboxylic acid reduction.**

| Enzyme class | Names | Common abbreviation |
|---|---|---|
| E.C. 1.2.1.30 | aryl-aldehyde dehydrogenase ($NADP^+$), aromatic acid reductase | CAR (carboxylic acid reductase) |
| E.C.1.2.1.31 | L-aminoadipate-semialdehyde dehydrogenase | AAR (α-aminoadipate reductase) |
| E.C.1.2.1.42 | Hexadecanal dehydrogenase (acylating) | FAR (fatty acyl-CoA reductase) |
| E.C.1.2.1.50 | Long-chain-fatty-acyl-CoA reductase | FAR (fatty acyl-CoA reductase) |
| E.C.1.2.1.80 | Long-chain acyl-[acyl-carrier-protein] reductase | AAR (fatty acyl-ACP reductase) |
| E.C.1.2.1.84 | Alcohol-forming fatty acyl-CoA reductase | FAR (fatty acyl-CoA reductase) |
| E.C. 1.2.99.6 | Carboxylate reductase, aldehyde oxidoreductase | AOR (aldehyde ferrodoxin oxidoreductase) |

## Conclusions

Compared to the vast majority of chemical routes towards the reduction of carboxylic acids carboxylate reductase enzymes allow for catalytic processes with perfect chemoselectivity: other functional groups than carboxylic acids remain unaffected. Most importantly, the aldehyde product is not reduced by these enzymes further, as proven in a number of biochemical studies with purified enzymes. In potential applications with e.g. whole cell systems, the challenge remains to trap the valuable, typically rather reactive aldehyde products from further reactions. Although many potential sources for carboxylate reductases were described, only a handful of gene and/or protein sequences are currently known. The lion's share of these enzymes remains to be explored and exploited for biotechnology.

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## Conflict of interests

*The authors declare no commercial or financial conflict of interest.*

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# 6 Whole-cell carboxylate reduction for the synthesis of 3-hydroxytyrosol

## FULL PAPERS

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# Whole-cell carboxylate reduction for the synthesis of 3-hydroxytyrosol

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3-Hydroxytyrosol (3-HT) is a phenolic antioxidant that has a number of beneficial effects on human health and is a valuable building block in the synthesis of various pharmaceuticals. Herein, we report a new method for the production of 3-HT through reduction of 3,4-dihydroxyphenylacetic acid. The reduction was performed in whole *Escherichia coli* BL21 (DE3) cells overexpressing carboxylic acid reductase from *Nocardia* and phosphopantetheinyl transferase from *E. coli.* An endogenous *E. coli* aldehyde reducing activity turned out to be highly efficient for further reduction of the aldehyde intermediate to the desired alcohol. The influence of different buffer components, cofactors, and cofactor recycling systems was investigated. A very economic combination of glucose, citrate, and air proved sufficient for recycling of the essential cofactors ATP and NAD(P)H. Selected crucial parameters were then further optimized within a "design of experiments" approach. Finally, first preparative-scale bioreductions resulted in pure 3-HT.

**Keywords:** Carboxylate reductase • 3-hydroxytyrosol (3-HT) • 3,4-dihydroxyphenylacetic acid (DOPAC) • whole cell biocatalysis • design of experiments (DoE)

## Introduction

Olive oil and wine contain 3-hydroxytyrosol (3-HT) – a phenolic compound with strong antioxidation power that was associated to a number of beneficial effects on human health.[1] In addition to the pronounced effects of 3-HT itself, the chemical structure is found as a building block in various pharmaceutically active compounds and can be used as a precursor for their synthesis. 3-HT is, for example, a building block in Vernakalant (Brinavess®), a drug used for the maintenance of the sinus rhythm of the heart.[2] Moreover, the structural element is found in reticuline, which is, in turn, a key component in metabolites in the sanguinarine/berberine and morphinan branch.[3]

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3-HT can be isolated from olives, olive leaves or other olive derived mixtures such as waste water from olive oil production. Despite these attractive sources, the described processes typically require expensive chromatographic steps.[4] Alternatively, tyrosol was described as a substrate for whole cell biotransformations which took advantage of hydroxylating activities of *Pseudomonas aeruginosa*[5] or *Serratia marcescens*.[6] In a similar approach, *Halomonas sp.* strain HTB24 was used, however, 3-HT was further oxidized to 3,4-dihydroxyphenylacetic acid.[7] In addition to the aforementioned approaches with limited insight on the catalyst(s) involved in product formation, a handful of enzyme catalyzed syntheses of 3-HT were described. The application of a commercial mushroom tyrosinase for tyrosol oxidation, e.g., yielded 3-HT but the process required ascorbic acid as additive to suppress further oxidation to the corresponding quinone.[8] Most recently, recombinant *Geobacillus thermoglucosidasius* phenol hydroxylase was used for tyrosol oxidation, however, the applicable substrate/product concentration was limited to 5 mM.[9] An alternative enzymatic method is based on β-glycosidase mediated hydrolysis of oleuropein at laboratory scale. Due to the hyperthermophilic origin of

the enzyme, the reaction proceeded at 70 °C.[10] Recently, also 2-phenylethanol was described as a substrate for 3-HT production with engineered toluene monooxygenase from *Pseudomonas mendocina* KR1 as a catalyst.[11] Apart from biocatalytic strategies, a number of chemical routes to 3-HT were described, e.g. from 3,4-dihydroxyphenylacetic acid via reduction with lithium aluminum hydride[12,4] as well as from its ester with lithium aluminum hydride,[13] sodium borohydride[14] or tetrabutylammonium borohydride.[15] The latter examples demonstrate that the reduction of carboxylic acids by chemical means requires strong reducing agents or a preceding transformation to more reactive derivatives. a possibility to reduce carboxylic acids selectively in aqueous systems is highly attractive for industrial applications. However, the literature is not very rich in reports that describe biotransformations from carboxylic acids to aldehydes and/or alcohols.

To our knowledge, the first report on carboxylate reductions appeared in 1959 in which the fungus *Polystictus versicolor* reduced a small number of aromatic carboxylic acids to the respective aldehydes and alcohols.[16] Subsequently, a moderate number of similar approaches using organisms, parts of organisms or cell extracts were described. In contrast, a range of aryl- and cinnamic aldehydes were prepared from their corresponding acids catalyzed by purified aryl-aldehyde:NADP-oxidoreductase from *Neurospora crassa.*[17]

A carboxylate reductase from *Nocardia* sp. NRRL 5646 was purified and characterized in the group of Rosazza *et al.*, including an investigation of the enzyme's substrate scope.[18] The gene coding for this enzyme was discovered and cloned for heterologous expression in *E. coli,*[19] however, a functional protein was only obtained after post-translational modification.[20] The same enzyme was expressed by another group in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to generate microbial strains for the production of vanillin.[21] Recently, another bacterial carboxylate reductase from *Mycobacterium marinum* was described with the so far unique ability to reduce fatty acids.[22] All these reductases appear to share a common pattern – they consist of 3 domains:

1) an adenylating domain to activate the carboxylate as an adenosyl phosphate at the expense of ATP,

2) a phosphopantethein binding site (which needs an additional enzyme to attach phosphopantethein at the expense of coenzyme a (CoA) to the enzyme); the cysteine of phosphopantethein attacks the carbonyl of the adenosyl phosphate to form a thioester, and

3) an NADPH dependent reductase domain that reduces the thioester and releases the aldehyde product.

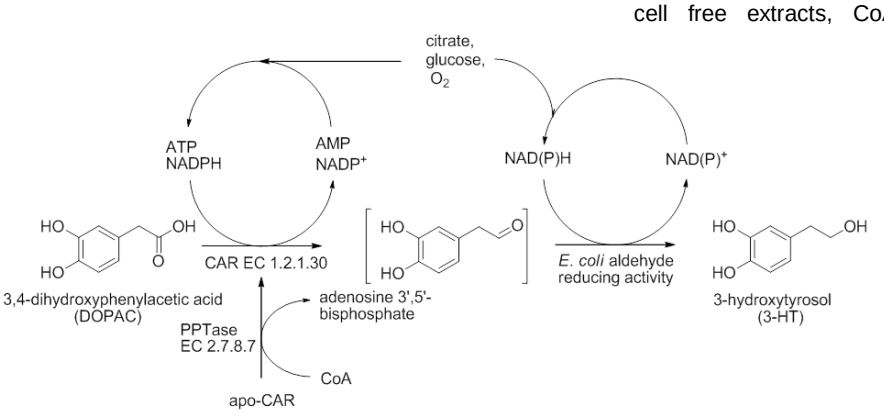
In addition to these carboxylate reductase activities, microbial reductions of carboxylic acids under anaerobic conditions have been described.[23] From all these biocatalysts only few were explored for their biotechnological potential so far.[20b,21,22]

Herein, we describe a new approach towards the preparation of 3-HT. Using a whole cell *E. coli* catalyst, we utilized a recombinant carboxylate reductase in combination with endogenous aldehyde reducing activity for the reduction of 3,4-dihydroxyphenylacetic acid (DOPAC) (see Scheme 1). In our system, all necessary cofactors (CoA, ATP and reducing equivalents) were finally provided and recycled by the whole cell system to generate 3-hydroxytyrosol from DOPAC, driven by citrate, glucose and air.

## Results

### Co-expression of carboxylate reductase and PPTase in *E. coli*

As the first step to explore carboxylate reductases for the reduction of 3,4-dihydroxyphenylacetic acid (DOPAC), we created three *E. coli* BL21 strains: one expressing a synthetically optimized gene sequence of *Nocardia* sp. CAR (*Ni*CAR), the second expressing *Ni*CAR and the phosphopantetheinyl transferase from *E. coli* (*Ec*PPTase) simultaneously, and the third (a control strain) harboring the two respective empty plasmids. CAR activity was verified by the reduction of vanillic acid to vanillin, using cell free extracts, CoA, ATP and NADPH as co-substrates. Subsequently, DOPAC was applied as a substrate under the same conditions and activity for both substrates was observed for the strain expressing both enzymes CAR and PPTase, whereas the strains expressing the single enzyme and the control strain did not exhibit significant reduction activity. Against all expectations based on literature and our own results with vanillic acid, we did not find any 3,4-dihydroxy-




**Scheme 1.** Substrates and key enzymes involved in the whole cell catalyzed cascade reaction from 3,4-dihydroxyphenylacetic acid (DOPAC) to 3-hydroxytyrosol (3-HT).

phenylacetaldehyde but exclusively the desired product 3-hydroxytyrosol.This suggests that, *E. coli* BL21 exhibits a high 3,4-dihydroxyphenyl-acetaldehyde reductase activity.

**Table 1.** Exploration of reaction conditions for whole cell bioreduction of DOPAC to 3-HT.[a]

| Entry | CoA [mM] | ATP [mM] | NADPH [mM] | Glucose [mM] | Toluene[24] [mM] | $O_2$ access | 3-HT [mM] |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 40 | 40 | 0 | 38 | No | 1.2 |
| 2 | 1 | 0 | 0 | 200 | 38 | No | 0.1 |
| 3 | 0 | 0 | 40 | 0 | 38 | No | 0.9 |
| 4 | 1 | 0 | 40 | 0 | 38 | No | 0.8 |
| 5 | 1 | 0 | 40 | 200 | 38 | No | 1.2 |
| 6 | 1 | 0 | 40 | 0 | 38 | Yes | 7.3 |
| 7 | 1 | 0 | 40 | 200 | 38 | Yes | 13.8 |
| 8 | 1 | 40 | 40 | 0 | 38 | Yes | 16.2 |
| 9 | 0 | 0 | 40 | 0 | 38 | Yes | 14.3 |
| 10 | 0 | 0 | 40 | 200 | 38 | Yes | 4.0 |
| 11 | 0 | 0 | 40 | 0 | 0 | Yes | 11.4 |

[a] Reaction conditions: Thawed cell paste in 50 mM potassium phosphate buffer pH 7.5 was either used without additional CoA or preincubated with 1 mM CoA for 1 h. 20 mM $MgCl_2$, 20 mM DOPAC, ATP, NADPH, glucose and toluene were added as specified in the matrix above. The reactions proceeded at 28 °C in an Eppendorf Thermomixer at 1000 rpm in 1.5 mL reaction tubes with open or closed lids.

**Table 2.** Effect of different buffer components on DOPAC reduction yield and recovery of substrate and product in 50 mM MES buffer at pH 6.0.

| Entry | $MgCl_2$ [mM] | DTT [mM] | EDTA [mM] | Glycerol [% v/v] | Relative conversion [%] | Relative mass balance[a] [%] |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 100 ± 6 | 76 ± 7 |
| 2 | 10 | 0 | 0 | 0 | 148 ± 13 | 48 ± 6 |
| 3 | 10 | 0 | 1 | 0 | 80 ± 8 | 103 ± 6 |
| 4 | 10 | 1 | 0 | 0 | 69 ± 13 | 97 ± 7 |
| 5 | 0 | 0 | 1 | 0 | 45 ± 17 | 102 ± 5 |
| 6 | 0 | 1 | 0 | 0 | 85 ± 21 | 45 ± 13 |
| 7 | 0 | 1 | 1 | 0 | 68 ± 5 | 98 ± 6 |
| 8 | 10 | 1 | 1 | 0 | 89 ± 11 | 95 ± 6 |
| 9 | 0 | 0 | 0 | 10 | 68 ± 8 | 100 ± 9 |
| 10 | 10 | 0 | 0 | 10 | 77 ± 10 | 107 ± 5 |
| 11 | 10 | 0 | 1 | 10 | 26 ± 3 | 109 ± 4 |
| 12 | 10 | 1 | 0 | 10 | 43 ± 15 | 106 ± 7 |
| 13 | 0 | 0 | 1 | 10 | 77 ± 8 | 108 ± 8 |
| 14 | 0 | 1 | 0 | 10 | 74 ± 7 | 105 ± 6 |
| 15 | 0 | 1 | 1 | 10 | 68 ± 12 | 99 ± 3 |
| 16 | 10 | 1 | 1 | 10 | 51 ± 3 | 100 ± 1 |

[a] Each reation was performed in quadruplicate

**Exploration of biotransformation parameters**

To define suitable reaction conditions, several reaction parameters were investigated: CoA (for the transformation of inactive apo-CAR into active holo-CAR), ATP (for DOPAC activation), NADPH (reduction equivalent), glucose (for NADPH regeneration), toluene (for cell permeabilization),[24] and the presence of molecular oxygen (for ATP and NADPH regeneration). In Table 1 several sets of conditions and the respective analytical yields of 3-HT are listed.

In precedent studies with purified CAR enzyme, the buffers were typically formulated with MgCl2, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), and glycerol and adjusted to pH 7.5.[20a] However, this pH value is inconsistent with the autooxidation properties of catechols at elevated pH.[25] The stability of DOPAC in potassium phosphate buffer was therefore investigated at various pH values by overnight incubation in the presence or absence of $MgCl_2$, DTT, EDTA, and glycerol. Up to pH 6.5 all samples remained colorless and the added amount of DOPAC was recovered. Higher pH caused losses but, notably, these losses were lower in the presence of $MgCl_2$, DTT, EDTA, and glycerol. Moreover, less browning was observed in 2-morpholinoethanesulfonic acid (MES) buffer than in potassium phosphate buffer (data not shown). Consequently, 50 mM MES buffer at pH 6.0 was used as a basis for all further bioconversions. Subsequently, the effect of all possible combinations of these additives on bioreduction yields of DOPAC was investigated separately and the results are summarized in Table 2.

Finally, the temperature dependence of DOPAC reduction was determined between 20 and 34 °C. The optimum was found in accordance to the literature at 28 °C.[20a]

**Cosubstrate preference, recycling and regeneration**

Having established suitable conditions for the reduction of DOPAC, the next aim was the exploration and minimization of nicotinamide cofactor requirement. The cofactor dependence of endogenous *E. coli* aldehyde reducing activity that reduces the intermediate 3,4-dihydroxyphenylacetaledhyde to 3-HT was yet unknown. Therefore, several combinations of nicotinamide cofactors were added to the biotransformations in different concentrations. NADP+ was added in combination with NADH in the hope that an enzymatic hydride transfer would occur.[26] However, in Figure 1 it is shown that combinations of $NADP^+$ and NADH were not useful for DOPAC reduction, because product formation was not significantly higher than in the case of no additional cofactor. The addition of one equivalent of NADPH was superior to that of a mixture of reduced cofactors, which gave comparable conversions to the addition of one equivalent of NADH. The highest concentrations of 3-HT were detected if two equivalents of reduced cofactors were added.

For cofactor recycling, we investigated the two enzymatic regeneration systems: glucose 1-dehydrogenase (GDH) for NAD(P)H and formate dehydrogenase (FDH) for NADH recycling. No additional nicotinamide cofactor was supplied to the respective experiments. Citrate and a combination of citrate and $NADP^+$[27] was utilized as cofactor regeneration systems.

In case of GDH/glucose, amounts of 5 mM 3-HT were detected after only five hours of reaction. However, standard deviations with this system were high, caused by the formation of an unidentified byproduct that partly overlapped with the 3-HT peak and prevented correct peak integration. Analytical yields of 3-HT in presence of FDH and sodium formate were on average 10% higher, but the most promising system appeared to be citrate: amounts of 7.7 mM of 3-HT were formed (77% conversion Figure 2).

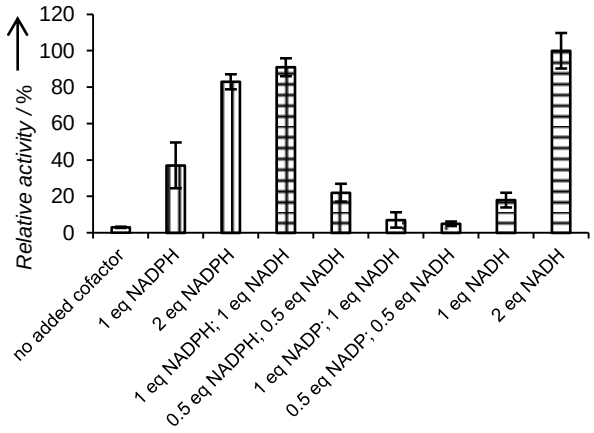



**Figure 1.** Two step reduction of DOPAC to 3-HT in the presence of nicotinamide cofactors in varying composition and amount. Each reaction was performed at least in triplicate.

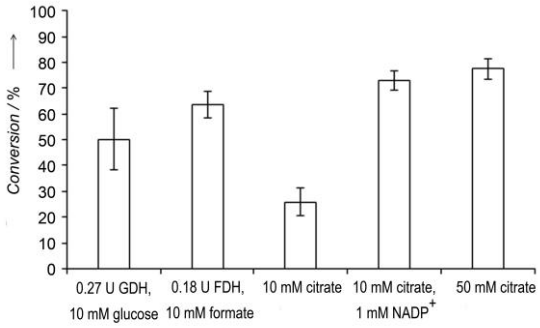



**Figure 2.** Cofactor recycling applied to CAR mediated DOPAC reduction. 10 mM DOPAC was incubated with thawed cell paste in 50 mM MES buffer containing 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT at pH 6.0 in the presence of cofactor regeneration components in open 1.5 mL tubes at 28 °C for 5 h. Each reaction was performed at least in triplicate.

Owing to the high number of parameters, which may influence the overall 3-HT yield, a "design of experiments" (DoE) approach was chosen for further optimization to minimize the amount of experiments. First, all known parameters were rated according to their expected influence. Second, six potential parameters were chosen as variables. Third, one set of parameters was defined as standard conditions (Table 3, entry 1) and appropriate reaction conditions were identified (e.g. revitalization time, reaction time, etc.). The standard deviation of bioconversions under these conditions was ±4%. All other conditions, except those specified in Table 3, were kept constant (e.g. cultivation and induction conditions, temperature, pH, etc.). After that, 17 different combinations of components concentrations as specified in Table 3 were investigated (entries 1-17) and analyzed using a polynomial model. Among the six tested parameters, only 4 of have been identified as being significant for the outcome of the reaction: DOPAC, citrate, glucose, and EDTA. No significant interactions among the tested reaction parameters were found. To properly describe the system, two more experiments were performed. a first set of parameters that was determined by the DoE software based on the previous results revealed lower activity than under standard conditions (Table 3, entry 18), but the second set of parameters given in Table 3, entry 19 (30 mM DOPAC, 67 mM citrate, 58 mM glucose, 1 mM DTT, 5.5 mM EDTA and 4.9 mM $MgCl_2$) appeared to be most efficient. Interestingly, the concentration differences compared to the standard conditions are not striking.

## Preparative scale bioreduction

Preparative scale bioconversions were performed as batch reactions in 100 mL volume using 10 and 30 mM of the substrate, respectively, (entry 4 and 19 in **Error! Reference source not found.**). Whereas the reaction mixture containing 30 mM DOPAC produced 1.2 mM of 3-HT within 20 h. 1.0 mM of 3-HT was produced within this time with the reaction mixture containing 10 mM DOPAC. However, after addition of new cell aliquots and cosubstrates, the 3-HT level did not increase further under the conditions described in entry 19. In contrast, the reaction with 10 mM DOPAC proceeded slowly to approximately 4 mM of 3-HT (39% conversion). The catechols were then extracted and purified to give 29.2 mg of pure 3-HT after a total bioconversion time 144 h (Figure 3). This corresponds to an overall yield of 19% and a productivity of approximately 2 mg $L^{-1}$ $h^{-1}$.

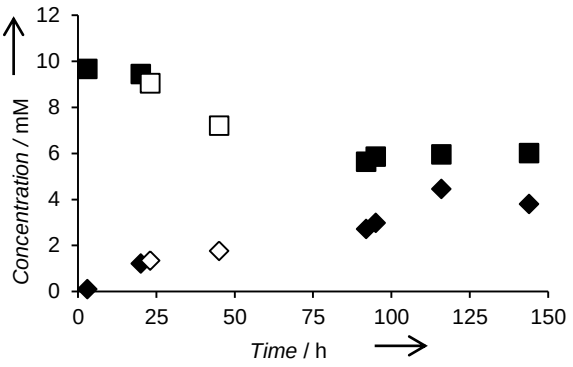



**Figure 3.** 3-HT formation in course of preparative scale reduction. ♦: 3-HT; ■: DOPAC . White diamonds and squares: addition of fresh aliquot of pre-incubated cells.

**Table 3.** Optimization of reaction conditions for whole cell bioreduction of DOPAC to 3-HT using the DoE methodology.[a]

| Entry | DOPAC [mM] | Citrate [mM] | Glucose [mM] | DTT [mM] | EDTA [mM] | $MgCl_2$ [mM] | Relative 3-HT concentration [%] |
|---|---|---|---|---|---|---|---|
| 1 | 30 | 55 | 55 | 5.5 | 5.5 | 5.5 | 100 ± 7 |
| 2 | 10 | 10 | 10 | 1 | 1 | 1 | 30 ± 1 |
| 3 | 10 | 10 | 10 | 10 | 1 | 10 | 30 ± 1 |
| 4 | 10 | 10 | 100 | 1 | 10 | 10 | 40 ± 5 |
| 5 | 10 | 10 | 100 | 10 | 10 | 1 | 38 ± 2 |
| 6 | 10 | 100 | 10 | 1 | 10 | 10 | 31 ± 1 |
| 7 | 10 | 100 | 10 | 10 | 10 | 1 | 25 ± 1 |
| 8 | 10 | 100 | 100 | 1 | 1 | 1 | 46 ± 1 |
| 9 | 10 | 100 | 100 | 10 | 1 | 10 | 44 ± 2 |
| 10 | 50 | 10 | 10 | 1 | 10 | 1 | 4 ± 3 |
| 11 | 50 | 10 | 10 | 10 | 10 | 10 | 3 ± 0 |
| 12 | 50 | 10 | 100 | 1 | 1 | 10 | 12 ± 4 |
| 13 | 50 | 10 | 100 | 10 | 1 | 1 | 62 ± 1 |
| 14 | 50 | 100 | 10 | 1 | 1 | 10 | 20 ± 4 |
| 15 | 50 | 100 | 10 | 10 | 1 | 1 | 21 ± 2 |
| 16 | 50 | 100 | 100 | 1 | 10 | 1 | 15 ± 3 |
| 17 | 50 | 100 | 100 | 10 | 10 | 10 | 9 ± 1 |
| 18 | 30 | 100 | 10 | 5 | 4.2 | 1.1 | 41 ± 8 |
| 19 | 30 | 67 | 58 | 1 | 5.5 | 4.9 | 147 ± 11 |

[a] Thawed and washed cell paste in 50 mM MES buffer pH 6.0 was preincubated with glucose and citrate for 1 h. DTT, EDTA, $MgCl_2$ and DOPAC were added as specified. The reactions proceeded for 21 h at 28 °C in an Eppendorf Thermomixer at 1000 rpm in 1.5 mL tubes with open lids. Each reaction was performed in triplicate.

## Discussion

Biobased routes to valuable products are highly desirable, however, for an industrially feasible synthetic process, the addition of expensive cosubstrates and cofactors must be reduced to a minimum and this may be partly achieved by using whole cell systems. *E. coli* appeared to be a suitable cell factory for 3-HT production, since it is fast growing, easy to handle and we and others[19] demonstrated that the BL21 (DE3) strain is capable to express the active form of carboxylate reductase (CAR) from *Nocardia iowensis* in the presence of overexpressed phosphopantetheinyl transferase. In the case of EC1.2.1.30 CAR mediated reductions, the supply of CoA, ATP and NADPH must be ensured. Arguing that ATP recycling would be more efficient in the presence of oxygen and glucose and that the cells may provide sufficient amounts of CoA for CAR activation by PPTase, several conditions were tested. Clearly, access of oxygen was beneficial and resulted in much higher 3-HT titers (Table 1 entries 6-11) than in experiments in closed vessels (Table 1, entries 1-5). The effect of additional CoA, glucose and toluene was, however, not pronounced under these conditions or could not clearly be attributed to these supplements. However, despite high product concentrations in entries 6-11, it needs to be stressed that a mass balance problem was observed: The amount of substrate and product detected by HPLC/MS was on average much lower than the initial amount of DOPAC substrate added (20 mM). In entry 9, for instance, the conversion of DOPAC appears to be 100% although only 16.2 mM 3-HT was recovered instead of 20 mM. The reaction mixtures had, moreover, developed orange-brownish color, which indicated a loss of substrate or product by degradation, for example, by autooxidation to quinones.[25] Therefore, it was necessary to optimize the reaction conditions, in particular, with respect to the pH. a substrate stability test revealed that DOPAC is fully recovered up to pH 6.5 and that whole cell biotransformation yields of 3-HT are equal at pH 6.0 and 6.5. All further experiments were thus performed at pH 6.0 in 50 mM MES buffer. As also the addition of buffer components such as salts and especially DTT is a considerable cost factor in a production process, the effect of $MgCl_2$, DTT, EDTA and glycerol on the reaction outcome and mass balance was evaluated. Omission of all additives and the addition of solely $MgCl_2$ or DTT resulted in sample browning and low recovery of substrate and product (Table 2, entries 1,2 and 6), but all other conditions allowed the recovery of material amounts of 20 mM. However, clear differences in carboxylate

reduction efficiency to 3-HT were observed (Table 2, entries 3-5 and 7-16) with an apparent optimum if $MgCl_2$, DTT, EDTA were present but not glycerol (Table 2, entry 8). This effect may be a result from undesired glycerol metabolism: the biomass that was used for these experiments had been harvested only four hours after induction with the intention to utilize metabolically active cells with high ATP production efficiency. In summary, 50 mM MES buffer at pH 6.0 supplemented with $MgCl_2$, DTT, EDTA solved the mass balance problem and was used for further experiments.

NADPH was reported to be the nicotinamide cofactor exclusively utilized by carboxylate reductase from *Nocardia* sp. NRRL 5646.[18a] We reasoned that also its oxidized form in combination with NADH – less costly compounds – may serve to promote the thioester reduction because whole cell systems are known to establish a cell internal cofactor equilibrium[26] which would constantly deliver NADPH to the CAR enzyme as well as the preferred cofactor to the endogenous enzyme responsible for 3,4-dihydroxyphenylacetaldehyde reduction. Surprisingly, highest product concentrations were observed upon addition of two equivalents of NADH. NADH is not only cheaper and more stable, but also cofactor recycling systems for NADH are typically more convenient to use, as compared to systems for NADPH.[28] Venkitasubramanian *et al.* had tackled NADPH supply by co-overexpressing GDH.[20a] Due to our own observations, we investigated both of the broadly used systems GDH/glucose as well as FDH/formate for cofactor recycling. In addition, citrate was explored as a substrate for the Krebs-cycle for simultaneous formation of ATP and reducing equivalents. Most conveniently, the reaction system supplied with citrate appeared to be the most efficient (Figure 2). Citrate as sacrificial substrate is not only significantly more economic than the addition of cofactor recycling enzymes, but also does not form any inconvenient byproducts like gluconic acid in the case of glucose oxidation.

Having collected information about several necessities of this whole cell bioreduction, the next aim was to maximize the substrate load and simultaneously minimize the concentration of additives.[29] The limits for CAR as the key catalyst for DOPAC reduction remained to be fully explored. At this point we had observed product concentrations of up to approximately 20 mM. By contrast, the substrate load was reported to be a major obstacle in bio-oxidative routes towards 3-HT, in which product inhibition allowed concentrations not higher than 5 mM[9] but typically even less.[11]

The optimization of one parameter at a time not only requires enormous numbers of experiments in multi-parameter systems but also leads to quasioptima. The design of experiments (DoE) methodology is utilized as a tool for systematic organization and evaluation of various factors affecting the performance of such systems.[30] For whole cell reduction of DOPAC to 3-HT, we chose six variable parameters that should be optimized first, in order to achieve highest possible product yields. Defined combinations of concentrations of substrate, citrate, glucose, DTT, EDTA and $MgCl_2$ were explored by keeping all other parameters constant. Each round of optimization was accompanied by a standard experiment (Table 3, entry 1) to allow better comparison. The results of three rounds of optimization are summarized in Table 3 and the conditions listed in entry 19 seemed to be a good starting point for a preparative scale reaction. However, an attempt to up-scale the DOPAC reduction under these conditions resulted in low conversions (data not shown). At conditions listed in **Error! Reference source not found.** entry 4 at a 100 mL reaction scale, the conversions after 23 h reaction time was only 25% of those obtained in the analytical scale experiments. We attributed these results partly to a disintegration of the biocatalyst by mechanical stress. However, addition of fresh cell paste together with additional citrate and glucose could not drive the reaction to completion. The reaction was terminated at 39% conversion. As DOPAC was present in the mixture, the product could not be selectively trapped, for example, with boric acid.[11] Moreover, simple extraction from basified aqueous solution was not an option in this case, owing to the instability of 3-HT at elevated pH. Instead, 3-HT was isolated in 19% overall yield (29.2 mg) by silica gel chromatography to homogeneity (>99%).

Further optimization of the reaction setup is still necessary to generate an economic process. a prerequisite to successful reaction engineering would be a deeper understanding of the applied biocatalyst and the analysis of several other parameters such as the effects of cultivation time on the activity of the ATP and NAD(P)H recycling machinery. The oxygen supply as well as the aldehyde reducing activity may become bottlenecks in course of further optimizations. Then, the identification of the 3,4-dihydroxyphenylacetaldehyde reducing activity would be necessary. The biocatalyst itself might also be further improved e.g. by steering the expression levels of *Ni*CAR and PPTase or by overexpression of other essential enzymes.

## Conclusion

In this study, we presented the first reductive enzyme catalyzed way to the natural antioxidant 3-hydroxytyrosol (3-HT).[32] The applied whole cell biocatalyst combines the reactivity of a carboxylate reductase with a number of endogenous reactions – including those for cofactor regeneration – to reduce the precursor 3,4-dihydroxy-phenylacetic acid in two steps to the corresponding alcohol. Product loss by quinone formation was avoided by carrying out the reaction at pH 6.0 and by tailoring the reaction medium. The “design of experiments” methodology was used for the simultaneous optimization of six critical parameters with a manageable number of experiments. On analytical level, we were able to detect full substrate conversion at 10 mM concentration under several conditions and up to 27.9 mM of 3-HT within 21 h using 30 mM of DOPAC. First preparative scale reactions allowed the isolation and purification of 3-HT in 19% overall yield and >99% purity. An extension of the “design of experiments” methodology including further

parameters, for example, from the biocatalyst preparation and the scale-up is envisaged and will certainly lead to further improvements.

## Experimental Section

**Materials and Equipment**

Ampicillin (Amp), CoA and 3,4-dihydroxyphenylacetic acid were purchased from Sigma Aldrich and acetonitrile (ACN) from J. T. Baker. ATP and NAD(P)(H) cofactors were purchased from Roche Diagnostics, Isopropyl β-D-1-thiogalactopyranoside (IPTG) from Biosynth and all other chemicals and buffer components were obtained from Carl Roth. GDH was obtained from DSM Innovative Synthesis BV and 1 mg corresponds to 2.7 U for $NADP^+$ and 3.4 U for $NAD^+$ reduction. Formate dehydrogenase (FDH) was used as cell-free extract of an *E. coli* Rosetta (DE3) containing the plasmid pRSF:FDH from *Candida boidinii* as described in [31] and 1.08 mg corresponds to 0.375 U for $NAD^+$ reduction. NMR spectra were recorded on a Bruker AVANCE III 300 spectrometer ($^1$H: 300.36 MHz; $^{13}$C: 75.53 MHz) and chemical shifts are referenced to residual protonated solvent signals as internal standard. For HPLC measurements, an Agilent Technologies 1200 Series equipped with G1379B degasser, G1312B binary pump SL, G1367C HiP-ALS SL autosampler, a G1314C VWD SL UV detector, G1316B TCC SL column oven and a G1956B mass-selective detector (MSD) were used. Samples were filtered through AcroPrep 96 Filter Plate (Pall Life Science) with 0.2 µm polypropylene membrane prior to analysis. The 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetaldehyde and 3-hydroxytyrosol were separated on a Chromolith Performance RP-18 endcapped 100-4.6 column at 30 °C by using a 95% aqueous eluent (0.1% formic acid) and 5% ACN at a flow of 1.2 mL $min^{-1}$ which was split to 0.8 mL $min^{-1}$ before themass-selective detection. Vanillic acid, vanillin and vanillyl alcohol were separated with 30% ACN content under otherwise identical conditions.

**Strain generation**

The genes coding for *Nocardia iowensis* NRRL 5646 CAR (EMBL accession no. Q6RKB1, gene sequence optimized for expression in *E. coli*) and PPT-ase entD from *E. coli* BL21 (DE3) (NCBI accession no. CAQ31055.1) were ordered at DNA2.0 (California, USA) in pJexpress-404 and 401 expression vectors, respectively. Plasmid DNA was retrieved according the protocol delivered with the genes and the sequence was verified by sequencing (LGC genomics). Electrocompetent *E. coli* BL21 (DE3) Gold cells were co-transformed with plasmid DNA of both constructs (1.13 µg of pJexpress-404-CAR and 0.11 µg of pJexpress-401-EcPPTase). Positives were selected on LB-plates supplemented with 100 µg $mL^{-1}$ ampicillin (Amp) and 50 µg $mL^{-1}$ kanamycin (Kan) after overnight incubation at 37°C.

**Cell cultivation**

A glycerol stock sample of *E. coli* BL21 (DE3) Gold:pJexpress-404-*Ni*CAR/pJexpress-401-*Ec*PPTase was used for inoculation of LB medium containing 100 µg $mL^{-1}$ Amp and 50 µg $mL^{-1}$ Kan. Standard cultivation was carried out and the main culture was induced with 1 mM IPTG at an $OD_{600}$ between 0.4 and 0.6. The protein expression phase proceeded for 4 h at 37 °C and 120 rpm. The cells were collected by centrifugation and washed by resuspending them in 50 mL of cold buffer (50 mM MES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EDTA, pH 6.0) prior to another centrifugation step for 10 min at 4°C and 2831 x *g*. Washing was repeated and all supernatants were discarded. Washed cells were dispersed in 50% glycerol, shock-frozen in liquid nitrogen and stored at -80 °C. Typically, the cells were used within a few days because the overall activity decreased significantly upon prolonged storage.

**Typical procedure for resting cell biotransformation**

Frozen cell suspensions were thawed and washed twice in reaction buffer (approximately 80 mg of wet cell pellet per sample) Unless otherwise stated, cofactor (typically 2 equiv.) or cofactor recycling components were added. The reactions were started by addition of DOPAC solution in 10 – 50 mM final concentration with adjusted pH (6.0) and proceeded in mixer incubator (Eppendorf Thermomixer) with open lid at 28 °C and 1000 rpm. The final $OD_{600}$ of the cells in the sample was about 120. To compensate for evaporation, the 1.5 mL reaction tubes were refilled to the 500 µL mark with water. Subsequently, the biomass was removed by centrifugation at 16 100 x *g* at RT for 5 min and the supernatants were filtered before HPLC analysis under conditions described above.

**Design of experiments (DoE)**

The reactions were performed as described above with the following modifications: All cells used for these experiments were frozen for 16 h. The thawed cells were pre-incubated with citrate and glucose for 1 h in order to revitalize their metabolic activity. The reaction time was 21 h. Planning of the experiments and analysis of the experimental data was performed using commercially available software (such as Design-Expert from Stat-Ease, Inc).

**Preparative scale 3-HT production**

An aliquot of frozen resting cells was thawed on ice, diluted with 50 mM MES buffer pH 6.0 (supplemented with 10 mM $MgCl_2$, 1 mM DTT and 1 mM EDTA) and centrifuged for 20 min at 4°C and 2831 x g. The cell pellet (3 g) was washed two more times at the same conditions, except the centrifugation time, which was 10. The cells were finally dispersed in 50 mM MES buffer pH 6.0. Stock solutions of sodium citrate and glucose were added to give concentrations of 10 mM and 100 mM based on the final reaction volume, respectively. a magnetic stirring bar was added and the samples were incubated for 1h at 28 °C and 90 rpm without lid. After that, DTT, EDTA and magnesium chloride were added to give final concentrations of 1 mM, 10 mM and 10 mM, respectively. Subsequently, DOPAC in MES buffer at pH 6.0 was added to give a final concentration of 10 mM in a total volume of 100 mL and the reaction was stirred at 28 °C and 100 rpm. The final $OD_{600}$ of the cells in this reaction was 12.3. Samples (200 µL) were withdrawn periodically, centrifuged for 10 min at RT and analyzed. Fresh portions of cells (3 g) were pre-incubated with citrate and glucose as described above and they were added after 23 and 45 h, respectively. After 144h, the reaction was stopped by centrifugation for 10 min at 4°C and 3836 x g. The pellet was washed with MES buffer three times in the same conditions. The pH of the combined supernatants was adjusted to 2.0 with concentrated HCl. Then, this aqueous layer was extracted with ethyl acetate three times. The combined

extracts were dried with sodium sulfate and the solvent was removed under reduced pressure at 40°C, giving a crude yield of 360 mg. The crude product was mixed with triethylamine (150 µL) to improve the separation of the acidic residues during flash chromatography on silica gel (Merck silica gel 60, 0.040-0.063 mm, 60Å) using dichloromethane/methanol 15:1 as the eluent. The product-containing fractions were pooled and the solvent was removed under reduced pressure to give 29.2 mg of a slightly brownish oil (19% overall yield). Product purity was confirmed by HPLC (>99%).$^{1}$H-NMR (300 MHz, $CD_3OD$, 25 °C, TMS): $\delta$ = 2.66 (t, 2H, $^{3}J$(H,H) = 7.2Hz; -C$H_2$-C$H_2$-OH), 3.67 (t, 2H, $^{3}J$(H,H) = 7.2Hz; -C$H_2$-C$H_2$-OH), 6.52 (dd, 1H, $^{3}J$(H,H) = 7.9Hz, $^{4}J$(H,H) = 1.8Hz; Ar-H5), 6.65 (d, 1H, $^{4}J$(H,H) = 1.8Hz; Ar-H3), 6.67 (d, 1H, $^{3}J$(H,H) = 8.0Hz; Ar-H6). $^{13}$C-NMR (75 MHz, $CD_3OD$, 25°C, TMS): $\delta$ = 39.7 (-C$H_2$-C$H_2$-OH), 64.6 (-C$H_2$-C$H_2$-OH), 116.3 (Ar-C3), 117.1 (Ar-C6), 121.2 (Ar-C5), 131.8 (Ar-C4), 144.6 (Ar-C1), 146.1 (Ar-C2).

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# 7 Method for preparation of 3-hydroxytyrosol

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## 7.1 Abstract

The invention relates to the preparation of 3-hydroxytyrosol (3-HT) from 3,4-dihydroxyphenylacetic acid (DOPAC) using a carboxylic acid reductase CAR.

## 7.2 Background of the invention

3-HT is a potent antioxidant found in olive leafs and olive oil, and is present in high abundance in olive mill waste waters. 3-HT has been associated with lower mortality and incidence of cancer in Mediterranean regions and has been attributed cardio-protective properties. It is used as a food additive. There has been therefore an increased interest in the manufacturing and commercialization of 3-HT as nutritional supplement.

For the reduction of carboxylic acids by chemical means, the acid has usually to be converted into a more reactive derivative, and strong reducing agents are required. There is a need to find an environmentally friendly, efficient and cost effective process in an aqueous system.

US 5795759 B and Li *et al.*, Journal of Bacteriology, 1997, 179, 3482-3487, disclose purification, characterization and properties of a CAR from *Nocardia* sp. Strain NRRL 5646 of the enzyme class EC 1.2.1.30. a number of carboxylic acids as substrates were tested for the ability of the CAR to reduce them to their corresponding aldehydes in in vitro experiments. CAR does not reduce an aldehyde to an alcohol, whereas intact whole cells of *Nocardia* strain NRRL 5646 reduce carboxylic acids first to the aldehyde and then to the corresponding alcohol.

US7056714B and He *et al.*, Applied and Environmental Microbiology, 2004, 70, 1974-1881, disclose the CAR from *Nocardia* sp. strain NRRL 5646 with its gene and amino acid sequence. The DNA sequence is listed at the Gen-Bank-EMBL database under accession code AY495697.

In vitro reduction of benzoate, vanillic acid and ferulic acid with the recombinant CAR resulted in the corresponding aldehydes. In vivo biotransformations of the same substrates with recombinant *E. coli* B21 strains resulted in the smooth conversion of carboxylic acids to aldehydes and subsequently partly also to alcohols. Reduction of aldehydes formed by CAR to alcohols by an endogenous *E. coli* ADH similar to that observed in *Nocardia* is relatively slow. Biochemical engineering approaches with the recombinant organism might be exploited to diminish this unwanted side reaction.

Venkitasubramanian *et al.*, The Journal of Biological Chemistry, 2007, 282, 478-485, disclose that the CAR from *Nocardia* sp. strain NRRL 5646 requires post-translational

phosphopantetheinylation by a PPTase in order to be transformed from an apo- to a holo-enzyme, with only the latter showing the CAR activity.

Venkitasubramanian *et al.*, Enzyme and Microbial Technology, 2008, 42, 130-137, disclose the use of recombinant *E. coli* BL21-CodonPlus (DE2)-RP/pPV2.85 that expresses car, npt and gdh, for the reduction of vanillic acid to vanillin.

## 7.3 Summary of the invention

There was a need for finding a suitable system for the preparation of 3-HT from DOPAC by biotransformation.

The data of the in vitro experiments of Li *et al.*, Journal of Bacteriology, 1997, 179, 3482-3487, in table 2, shows the conversion for the following substrates in relation to the conversion of benzoate, with the conversion of benzoate set to 100% relative activity:

| | Relative Activity |
|---|---|
| Benzoic acid | 100% |
| 3-hydroxylbenzoic acid | 77% |
| 4-hydroxylbenzoic acid | 6% |
| phenylacetic acid | 43% |

This means, that especially a hydroxy residue in 4 position reduces the rate of conversion considerably. The skilled person would expect, that the relative activity for DOPAC as substrate would be (0.77 x 0.06 x 0.43) = 0.02, i.e. 2%, which is rather low.

The teaching of the prior art disclosing the application of CAR of *Nocardia* sp. is directed to the application of CAR for the reduction of carboxylic acids to their corresponding aldehydes. The subsequent reduction, i.e. the reduction of the aldehyde to its corresponding alcohol, is seen as an unwanted side reaction, for which He *et al.*, Applied and Environmental Microbiology, 2004, 70, 1974-1881, on page 1880 right column end of third last paragraph, suggest expressly the possibility of exploitation of biochemical engineering approaches with the recombinant organism in order to diminish this unwanted side reaction.

Venkitasubramanian *et al.*, Enzyme and Microbial Technology, 2008, 42, 130-137, disclose on page 134 end of first paragraph in left column, that the mass balance for the reduction by whole, resting cells of respective *E. coli* constructs of vanillic acid to vanillin by exogenous CAR and subsequently to vanillyl alcohol by an endogenous *E. coli* ADH was essentially 100%, as it was for the reduction of benzoic acid to benzyl alcohol, but ferulic acid was never completely reduced with mixtures of the starting acid, coniferyl aldehyde and coniferyl alcohol obtained at all times. This suggests that the carboxy residue should be directly connected with the phenyl ring, and not via intermediate carbon atoms. Further it is assumed on page 136 in the right column middle of first paragraph that the rate of reduction of vanillic acid by holo-CAR is much faster than the rate of reduction of the aldehyde vanillin by endogenous ADH.

The skilled person, in an attempt to find a suitable biotransformation system for DOPAC to 3-HT, would be dissuaded by the data presented in the prior art to use the disclosed CAR of *Nocardia* sp. in *E. coli* for this task: firstly, one would expect to have a low conversion rate in the first reduction step due to the substitution pattern of DOPAC, secondly, one would expect to end with a product mixture of the substrate, the acid, the intermediate, the aldehyde, and the desired product, the alcohol. Also the conversion rate of the endogenous ADH of *E. coli* was assumed to be lower relative to the conversion rate of CAR for the reduction of vanillic acid to vanillin.

Unexpectedly, against this teaching of prior art, a biotransformation system for the reduction of DOPAC to 3-HT based on a CAR of the enzyme class EC 1.2.1.30 was found, that is not only able to reduced DOPAC to 3-HT, but in all experiments it was not even possible to detect the intermediate aldehyde. This high efficiency not only for the first reduction step from the carboxylate to the aldehyde, but also for the second reduction step from the aldehyde to the alcohol, was unexpected especially in view of the disclosure of the prior art.

## 7.4 Detailed description of the invention

### 7.4.1 Abbreviations

| | |
|---|---|
| AADH | aryl-aldehyde dehydrogenase ($NADP^+$), EC 1.2.1.30 |
| ACPS | holo-[acyl-carrier-protein] synthase, EC 2.7.8.7 |
| acpS | gene coding for CgPPTase |
| ADH | alcohol dehydrogenase |
| BLAST | Basic Local Alignment Search Tool of NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi |
| BRENDA | database compiling enzymes and enzyme classes, accessible under http://www.brenda-enzymes.org/, published by Department of Bioinformatics and Biochemistry, Institute for Biochemistry and Biotechnology Technische Universität Braunschweig, Langer Kamp 19B, 38106 Braunschweig, Germany |
| CAR | carboxylic acid reductase |
| car | gene coding for CAR |
| CgPPTase | 4'-phosphopantetheinyl transferase of Corynebacterium glutamicum |
| DNA2.0 | DNA2.0, Inc., Menlo Park, USA, www.dna20.com |
| DOPAC | 3,4-dihydroxyphenylacetic acid |
| DTT | 1,4-dithiothreitol |
| EDTA | Ethylene diamine tetra acetic acid |
| *E. coli* | *Escherichia coli* |

| | |
|---|---|
| *Ec*PPTase | 4'-phosphopantetheinyl transferase of *E. coli* |
| EMBL | European Molecular Biology Laboratory, Heidelberg, Germany, http://www.embl.de/ |
| EMBL-EBI | European Molecular Biology Laboratory - European Bioinformatics Institute, Hinxton, UK, http://www.ebi.ac.uk/ |
| entD | gene coding for EcPPTase |
| FDH | formate dehydrogenase |
| fdh | gene coding for fdh |
| GDH | glucose 1-dehydrogenase (DSM Innovative Synthesis BV, Geleen, The Netherlands, www.dsm.com) |
| gdh | gene coding for GDH |
| 3-HT | 3-hydroxytyrosol |
| IUBMB | International Union of Biochemistry and Molecular Biology, http://www.iubmb.org/ |
| NCBI | National Center for Biotechnology Information, Bethesda, USA, http://www.ncbi.nlm.nih.gov/ |
| NC-IUBMB | Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, http://www.chem.qmul.ac.uk/iupac/jcbn/ |
| Novagene® | Merck Milipore, Merck KGaA, Darmstadt, Deutschland, www.merckmillipore.de |
| NPT | *Nocardia* sp. phosphopantetheinyl transferase |
| npt | gene coding for NPT |
| OD | Optical Density |
| PPTase | 4'-phosphopantetheinyl transferase |
| U | Unit is abbreviated with "U" for the purpose of this invention, if not otherwise stated |

AADH and CAR are used synonymously in this specification for an enzyme of the enzyme class EC 1.2.1.30.

ACPS and PPTase are used synonymously in this specification for an enzyme of the enzyme class EC 2.7.8.7.

The enzyme class EC is defined by the NC-IUBMB, which is accessible in the internet via http://www.chem.qmul.ac.uk/iubmb/enzyme/, and is also compiled e.g. in the database BRENDA.

The terms "alignment, "conserved substitution", "identity", "homology" and "similarity" are used as defined in BLAST:

alignment — The process or result of matching up the nucleotide or amino acid residues of two or more biological sequences to achieve maximal levels of identity and, in the case of amino acid sequences, conservation, for the purpose of assessing the degree of similarity and the possibility of homology.

conserved substitution — A change at a specific position of an amino acid or, less commonly, DNA sequence that preserves the physico-chemical properties of the original residue or achieves a positive score in the governing scoring matrix.

identity or sequence identity — The extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in an alignment, often expressed as a percentage identity of the amino acid residues, based on the total number of amino acid residues of the amino acid sequence. If the two sequences, which are being compared, have a different number of amino acid residues, the percentage is based on the number of the shorter amino acid sequence.

homology — Similarity attributed to descent from a common ancestor. Homologous biological components (genes, proteins, structures) are called homologs. Often the term homolog is used interchangeably with the term variant, meaning rather a certain degree of identity between two sequences without implying a common ancestor. Therefore within the meaning of the invention, the term variant of a sequence means a sequence with a certain degree of identity to the sequence, if not otherwise stated.

similarity — The extent to which nucleotide or protein sequences are related. Similarity between two sequences can be expressed as percent sequence identity and/or percent positive substitutions.

Unless otherwise stated, sequence identity, similarity and positives values provided herein refer to the value obtained using the BLAST suite of programs using default parameters, a description is found in Altschul *et al.*, J. Mol. Biol., 1990, 215, 403-410 and in "The NCBI Handbook" (Internet), editors J. McEntyre and J. Ostell, Bethesda (MD): National Center for Biotechnology Information (US); 2002-, and using the program version BLAST 2.2.27+, if not otherwise stated.

The term "sequence" can mean a nucleotide sequence, which constitutes a polynucleotide, or an amino acid sequence, which constitutes a peptide. Nucleotide sequences are found e.g. under the respective accession no. in the EMBL Nucleotide Sequence Database of the EMBL-EBI, or in the Nucleotide database of the NCBI; and amino acid sequences are found e.g. under the respective accession no. in the UniProt database of the EMBL-EBI, or in the Protein database of the NCBI; if not otherwise stated.

"Heterologous nucleic acid sequence" or "nucleic acid sequence heterologous to a host" means a nucleic acid sequence which encodes e. g. an expression product such as a polypeptide that is foreign to the host ("heterologous expression" or "heterologous product") e.g. a nucleic acid sequence originating from a donor different from the host or a chemically synthesized nucleic acid sequence which encodes e. g. an expression product such as a polypeptide that is foreign to the host. In a similar way, "heterologous protein" means a protein that is foreign to the host.

"Foreign to the host" can mean, that the nucleic acid or protein originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition or genomic locus by deliberate human intervention.

A heterologous nucleic acid sequence as referred herein encompasses also nucleic acids, which are codon optimized for the host according to the codon usage of the host.

Exogenous DNA refers to any deoxyribonucleic acid that originates outside of the organism of concern or study.

The terms "host", "host cell" and "recombinant host cell" are used interchangeably herein to indicate a cell, which contains or shall contain a vector or an isolated nucleic acid sequence, and supports the replication and/or expression of the vector or of the nucleic acid. It is understood that such terms refer not only to the particular subject cell but also to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, plant, amphibian, or mammalian cells.

A "vector", "vector expressible in a host" or "expression vector" are used interchangeably and is a polynucleic acid construct that is used in transfection or transformation of a host cell and into which a polynucleotide can be inserted, it is generated recombinantly or synthetically, with a series of specified polynucleic acid elements that permit transcription of a particular nucleic acid sequence in a host cell. Typically, this vector includes a transcriptional unit comprising a particular nucleic acid sequence to be transcribed operably linked to a promoter. Vectors are often replicons. a vector expressible in a host can be e. g. an autonomously or self-replicating plasmid, a cosmid, a phage, a virus or a retrovirus.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

In molecular biology, the process by which cells take up exogenous DNA from the outside is called "transformation". Bacteria need to be in a certain physiological state to successfully take up exogenous DNA, which is described as one of "competence". Some bacteria are naturally competent, but usually only for a brief time at a certain stage of their growth cycle. Bacteria can

also be made competent through a variety of chemical treatments including exposure to calcium ions, or a mixture of polyethylene glycol and dimethylsulfoxide, which make the cell membrane more permeable, leading to the uptake of the exogenous DNA. Another treatment method is the utilization of electricity as the membrane-permeabilizing agent (electroporation or electro transformation). Finally, liposome-mediated transformation can be used. In this method, DNA is coated with lipid. Fusion of this lipid and the membrane lipid can occur, facilitating the entry of DNA.

"Transduction" is usually used for the process of injection of foreign DNA by a virus into the host bacterium.

"Transfection" is the process of deliberately introducing nucleic acids into cells. The term is often used for the introduction of foreign DNA into eukaryotic cells, notably for non-viral methods.

In microbiology, genetics, cell biology and molecular biology, "competence" is the ability of a cell to take up extracellular DNA from its environment.

Within the meaning of the invention and if not otherwise stated, the terms "transformation", "transformed" or "introducing a nucleic acid into a host cell" are used interchangeably and denote any process wherein an extracellular nucleic acid like a vector, with or without accompanying material, enters a host cell. The term "cell transformed" or "transformed cell" means the cell or its progeny into which the extracellular nucleic acid has been introduced and thus harbors the extracellular nucleic acid. The nucleic acid might be introduced into the cell so that the nucleic acid is replicable either as a chromosomal integrant or as an extra chromosomal element. Transformation of appropriate host cells with e. g. an expression vector can be accomplished by well-known methods such as microinjection, electroporation, particle bombardment or by chemical methods such as Calcium phosphate-mediated transformation, described e. g. in Maniatis *et al.* 1982, Molecular Cloning, a laboratory Manual, Cold Spring Harbor Laboratory, in Ausubel *et al.* 1994, Current protocols in molecular biology, John Wiley and Sons, or in "Molecular Cloning: a laboratory Manual, Third edition (3 volume set)", Cold Spring Harbor Laboratory Press, 2001.

"Nucleic acid", "nucleic acid sequence" or "polynucleotide" are used interchangeably and together with "isolated and purified nucleic acid or nucleic acid sequence or polynucleotide", as referred in the present invention, might be DNA, RNA, or DNA/RNA hybrid. Unless otherwise indicated, the terms include reference to the specified sequence as well as the complementary sequence thereof. In case the nucleic acid sequence is located on a vector it is usually DNA. DNA which is referred to herein can be any polydeoxynuclotide sequence, including, e.g. double-stranded DNA, single-stranded DNA, double-stranded DNA wherein one or both strands are composed of two or more fragments, double-stranded DNA wherein one or both strands have an uninterrupted phosphodiester backbone, DNA containing one or more single-stranded portion(s) and one or more double-stranded portion(s), double-stranded DNA wherein the DNA strands are fully complementary, double-stranded DNA wherein the DNA strands are only partially complementary, circular DNA, covalently- closed DNA, linear DNA, covalently cross-linked DNA, cDNA, chemically synthesized DNA, semi-synthetic DNA, biosynthetic DNA, naturally-isolated DNA, enzyme-digested DNA, sheared DNA, labeled DNA, such as radio labeled DNA and fluorochrome-labeled DNA, DNA containing one or more non-naturally occurring species of nucleic acid. DNA

sequences can be synthesized by standard chemical techniques, for example, the phosphotriester method or via automated synthesis methods and PCR methods. The purified and isolated DNA sequence may also be produced by enzymatic techniques.

RNA which is referred to herein can be e.g. single-stranded RNA, cRNA, double- stranded RNA, double stranded RNA wherein one or both strands are composed of two or more fragments, double-stranded RNA wherein one or both strands have an uninterrupted phosphodiester backbone, RNA containing one or more single-stranded portion(s) and one or more double-stranded portion(s), double-stranded RNA wherein the RNA strands are fully complementary, double-stranded RNA wherein the RNA strands are only partially complementary, covalently cross-linked RNA, enzyme digested RNA, sheared RNA, mRNA, chemically-synthesized RNA, semi-synthetic RNA, biosynthetic RNA, naturally isolated RNA, labeled RNA, such as radiolabeled RNA and fluorochrome-labeled RNA, RNA containing one or more non-naturally occurring species of nucleic acid.

Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

With "variants" or "variants of a sequence" is meant a nucleic acid sequence that vary from the reference sequence by nucleic acid substitutions, preferably by conservative nucleic acid substitution, whereby one or more nucleic acids are substituted by another with same characteristics. Variants encompass as well degenerated sequences, sequences with deletions and insertions, as long as such modified sequences exhibit the same function (functionally equivalent) as the reference sequence.

The terms "polypeptide", "peptide", "protein", "polypeptidic" and "peptidic" are used interchangeably herein to refer to a polymer of amino acid residues connected to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term "isolated and purified nucleic acid sequence" refers to the state in which the nucleic acid sequence will be free or substantially free of material with which they are naturally associated such as other nucleic acids with which they are found in their natural environment, or the environment in which they are prepared (e. g. cell culture) when such preparation is by recombinant technology practiced in vitro or in vivo.

The "origin of replication" (also called the replication origin) is a particular sequence in a genome at which replication is initiated. This can either involve the replication of DNA in living organisms such as prokaryotes and eukaryotes, or that of DNA or RNA in viruses, such as double-stranded RNA viruses. DNA replication may proceed from this point bidirectionally or unidirectionally.

"Promoter" as used herein refers to a nucleic acid sequence that regulates expression of a transcriptional unit. a "promoter region" is a regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence.

Within the promoter region will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase such as the putative -35 region and the Pribnow box.

"Signal sequence" or "signal peptide sequence" refers to a nucleic acid sequence which encodes a short amino acid sequence (i.e., signal peptide) present at the NH2-terminus of certain proteins that are normally exported by cells to non-cytoplasmic locations (e.g., secretion) or to be membrane components. Signal peptides direct the transport of proteins from the cytoplasm to non-cytoplasmic locations.

"Translation initiation region" is a signal region which promotes translation initiation and which functions as the ribosome binding site such as the Shine Dalgarno sequence.

"Terminator", "transcription terminator" and "transcription termination region" are used interchangeably and indicate a section of genetic sequence that marks the end of gene or operon on genomic DNA for transcription. It causes RNA polymerase to terminate transcription. The transcription termination region is usually part of a transcriptional unit and increases the stability of the mRNA.

"Transcriptional unit" as used herein refers to a nucleic acid sequence that is normally transcribed into a single RNA molecule. The transcriptional unit might contain one gene (monocistronic) or two (dicistronic) or more genes (polycistronic) that code for functionally related polypeptide molecules.

A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a signal sequence is operably linked to DNA for a protein if it is expressed as a preprotein that participates in the secretion of the protein; a promoter is operably linked to a coding sequence if it affects the transcription of the sequence; or a translation initiation region such as a ribosome binding site is operably linked to a nucleic acid sequence encoding e. g. a polypeptide if it is positioned so as to facilitate translation of the polypeptide. Linking can be accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

Subject of the invention is a method for the preparation of 3-hydroxytyrosol by a reaction, the reaction comprises an enzymatic reduction of 3,4-dihydroxyphenyl acetic acid,

wherein the enzymatic reduction is done using two enzymes, a carboxylic acid reductase CAR and a 4'-phosphopantetheinyl transferase PPTase;

wherein

CAR comprises each of the three amino acid sequences SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5 with 70 to 100% identity;

PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14, each with 70 to 100% identity respectively.

### 7.4.2 List of Sequences

SEQ ID NO: 1 to 6 and 10 are derived from SEQ ID 7.

SEQ ID NO: 1: position 90 to 535 of SEQ ID NO: 7

SEQ ID NO: 2: position 90 to 544 of SEQ ID NO: 7

SEQ ID NO: 3: position 661 to 693 of SEQ ID NO: 7

SEQ ID NO: 4: position 658 to 704 of SEQ ID NO: 7

SEQ ID NO: 10: position 655 to 730 of SEQ ID NO: 7

SEQ ID NO: 5: position 789 to 1080 of SEQ ID NO: 7

SEQ ID NO: 6: position 750 to 1094 of SEQ ID NO: 7

SEQ ID NO: 7: UniProt database of the EMBL-EBI, EMBL accession no. Q6RKB1, amino acid sequence of CAR from *Nocardia* sp. strain NRRL 5646

SEQ ID NO: 8: EMBL Nucleotide Sequence Database of the EMBL-EBI, EMBL accession no. AY495697, nucleotide sequence of CAR gene of *Nocardia* iowensis, registered as "*Nocardia* sp. NRRL 5646 ATP/NADPH-dependent carboxylic acid reductase (car)" and translating in sequence of SEQ ID NO: 7

SEQ ID NO: 9: this is a SEQ ID NO: 8, which is codon optimized for *E. coli*

SEQ ID NO: 11: Nucleotide database of the NCBI, Escherichia coli BL21 (DE3), complete genome, accession no. AM946981.2 region 569939 to 570568, nucleotide sequence of a EcPPTase gene in its complementary form

SEQ ID NO: 12: Protein database of the NCBI, phosphopantetheinyl transferase, subunit of enterobactin synthase multi enzyme complex [*Escherichia coli* BL21 (DE3)], accession no. CAQ31055.1, amino acid sequence of a *Ec*PPTase

SEQ ID NO: 13: Nucleotide database of the NCBI, *Corynebacterium glutamicum* ATCC 13032, NC_003450, region 2634745 to 2635152, nucleotide sequence of a *Cg*PPTase gene in its complementary form

SEQ ID NO: 14: Protein database of the NCBI, 4'-phosphopantetheinyl transferase [*Corynebacterium glutamicum* ATCC 13032], NCBI Reference Sequence: NP_601692, amino acid sequence of a CgPPTase

SEQ ID NO: 15: Gene sequence of plasmid pJexpress-404-9-CAR

SEQ ID NO: 16: Gene sequence of plasmid pJexpress-401-11-EcPPTase

Primers:

SEQ ID NO: 17: Primer 1

SEQ ID NO: 18: Primer 2

SEQ ID NO: 19: Primer 3

SEQ ID NO: 20: Primer 4

SEQ ID NO: 21: Primer 5

SEQ ID NO: 22: Primer 6

SEQ ID NO: 23: Primer 7

SEQ ID NO: 24: Primer 8

SEQ ID NO: 1 and SEQ ID NO: 2 represent the adenylating domain of CAR, where AMP is bound.

SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 10 represent the phosphopantetheinyl binding site of CAR.

SEQ ID NO: 5 and SEQ ID NO: 6 represent the thioester reductase domain of CAR, where NADPH is bound.

CAR is classified in the literature to be an enzyme that catalyzes the reaction, which enzymes of the enzyme class EC 1.2.99.6 or of the enzyme class EC 1.2.1.30 catalyze.

According to IUBMB Enzyme Nomenclature and for the purpose of this invention, enzyme class EC 1.2.1.30 is assigned to enzymes, that have the accepted name "aryl-aldehyde dehydrogenase ($NADP^+$)".

They catalyze the reaction:

"an aromatic aldehyde + $NADP^+$ + AMP + diphosphate + $H_2O$ =

an aromatic acid + NADPH + H+ + ATP"

Other names are "aromatic acid reductase" and "aryl-aldehyde dehydrogenase (NADP)". Systematic name is "aryl-aldehyde: $NADP^+$ oxidoreductase (ATP-forming)". The CAS registry number is 9074-94-6.

According to IUBMB Enzyme Nomenclature and for the purpose of this invention, enzyme class EC 1.2.99.6 is assigned to enzymes, that have the accepted name: carboxylate reductase.

They catalyze the reaction:

"an aldehyde + acceptor + $H_2O$ = a carboxylate + reduced acceptor"

Other name is "aldehyde:(acceptor) oxidoreductase".

Systematic name is "aldehyde:acceptor oxidoreductase".

IUBMB enzyme Nomenclature gives a comments: "A tungsten protein. Methyl viologen can act as acceptor. In the reverse direction, non-activated acids are reduced by reduced viologens to aldehydes, but not to the corresponding alcohols." The CAS registry number ir 125008-36-8.

PPTase catalyzes the reaction, which enzymes of the enzyme class EC 2.7.8.7. catalyze.

According to IUBMB Enzyme Nomenclature and for the purpose of this invention, enzyme class EC 2.7.8.7 is assigned to enzymes, that have the accepted name "holo-[acyl-carrier-protein] synthase".

They catalyze the reaction:

"CoA-[4'-phosphopantetheine] + apo-[acyl-carrier protein] =

adenosine 3',5'-bisphosphate + holo-[acyl-carrier protein]"

Other names are acyl carrier protein holo protein (holo-ACP) synthetase; holo-ACP synthetase; coenzyme A:fatty acid synthetase apo enzyme 4'-phosphopantetheine transferase; holo synthase; acyl carrier protein synthetase; holo-ACP synthase; PPTase; AcpS; ACPS; acyl carrier protein synthase; P-pant transferase; CoA: apo-[acyl-carrier-protein] pantetheinephosphotransferase.

Systematic name is "CoA-[4'-phosphopantetheine]:apo-[acyl-carrier protein] 4'-pantetheinephosphotransferase". The CAS registry number is 37278-30-1.

CAR and PPTase are polypeptides. The scope of CAR and of PPTase within the meaning of the invention comprises "functional equivalents". Within the context of the present invention, functional equivalents of CAR and of PPTase are polypeptides which differ from CAR and of PPTase but which still possess the desired biological activity such as substrate specificity, for example. Thus, "functional equivalents" mean, for example, enzymes which reduce DOPAC to 3-HT and which have at least 50%, preferably at least 60%, more preferably at least 75%, even more preferably at least 90%, of the activity of an enzyme having the amino acid sequence listed in SEQ ID NO 7 or 12 respectively. Functional equivalents are also preferably stable between pH 4 to 10. They preferably possess a pH optimum between pH 5 and 8. They preferably possess a temperature optimum in the range from 20° C to 80° C. Functional equivalents thus comprise e.g. the mutants obtainable by one or more amino acid additions, amino acid substitutions, amino acid deletions and/or or amino acid inversions, it being possible for said alterations to occur in any sequence position, as long as they result in a mutant having the property profile of the invention. Functional equivalence also exists, in particular, when the reactivity patterns of the mutant and the unaltered enzyme with the amino acid sequence SEQ ID NO: 7 or 12 respectively, agree qualitatively, i.e. DOPAC is reduced to 3-HT at different rates.

Functional equivalents in the above sense are also "precursors" of the polypeptides described and also "functional derivatives" and "salts" of said polypeptides.

In this context, "precursors" are natural or synthetic precursors of the polypeptides with the desired reduction activity.

The expression "salts" means both salts of carboxyl groups and/or of amino groups of the polypeptides of the invention. Salts of carboxyl groups can be prepared in a manner known per se and comprise inorganic salts such as sodium, calcium, ammonium, iron and zinc salts as well as salts with organic bases, for example amines, such as triethanolamine, arginine, lysine, piperidine and the like. Salts of amino groups are for example salts with mineral acids such as hydrochloric acid or sulfuric acid and salts with organic acids such as acetic acid and oxalic acid.

"Functional derivatives" of polypeptides of the invention are covalently modified polypeptides of the invention, and may be prepared with the aid of known techniques e.g. by modification of functional side groups of the polypeptide such as amino side groups or hydroxy side groups, or by modification of the N-terminus or C-terminus. Modification is done preferably with protecting groups, which are commonly used in polypeptide chemistry and which are known to the skilled person. Functional derivatives of this kind comprise, for example, aliphatic esters of carboxylic acid groups, amides of carboxylic acid groups, which amides are obtainable by reacting with ammonia or with a primary or secondary amine; N-acyl derivatives of free amino groups, which derivatives are prepared by reacting the free amino groups with acyl groups; or O-acyl derivatives of free hydroxyl groups, which derivatives are prepared by reacting the free hydroxyl groups with acyl groups.

Functional equivalents naturally also comprise polypeptides which are available from other organisms than *E. coli* and also naturally occurring variants. For example, areas of homologous sequence regions can be established by sequence comparison and equivalent enzymes can be determined on the basis of the specific guidelines of the invention.

Functional equivalents are moreover fusion proteins which contain any of the abovementioned polypeptide sequences or functional equivalents derived therefrom and at least one further heterologous sequence which is functionally different therefrom and is covalently linked, preferably N -terminally or C-terminally (i.e. without any substantial reciprocal functional impairment of the fusion protein moieties). Non limiting examples of such heterologous sequences are signal peptides or enzymes, for example.

"Functional equivalents" which are comprised in the invention are variants of the specifically disclosed sequences. Said variants comprise an amino acid sequence with 70 to 100% identity, preferably with 80 to 100% identity, more preferably with 90 to 100% identity, even more preferably with 95 to 100% identity, especially with 100% identity, with the amino acid sequence with which it is compared.

Therefore within the meaning of the invention, the term variant of a sequence means preferably a sequence with a certain degree of identity to the sequence.

In the case of a possible protein glycosylation, functional equivalents comprise proteins of the above described type in deglycosylated or glycosylated form and also modified forms which can be obtained by altering the glycosylation pattern. Variants of the proteins or polypeptides of the invention may be generated by mutagenesis, for example by point mutation or truncation of the protein.

Variants of the proteins of the invention may be identified by screening combinatorial libraries of mutants such as truncation mutants. For example, a variegated library of protein variants may be

generated by combinatorial mutagenesis at the nucleic acid level, for example by enzymatically ligating a mixture of synthetic oligonucleotides.

There are a large number of methods which may be used for preparing libraries of potential variants from a degenerate oligonucleotide sequence. a degenerate gene sequence maybe synthesized chemically in a DNA synthesizer and the synthetic gene may then be ligated into a suitable expression vector. Using a degenerate set of genes makes it possible to prepare all the sequences in a mixture which encode the desired set of potential protein sequences. Processes for synthesizing degenerate oligonucleotides are known to the skilled person (e.g. Narang *et al.*, Tetrahedron, 1983 39, 3-22; Itakura *et al.*, Annu. Rev. Biochem., 1984, 53, 323-356; Itakura *et al.*, Science, 1984, 198, 1056-1063; Ike *et al.*, Nucleic Acids Res., 1983, 11, 477-488).

A plurality of techniques for screening gene products of combinatorial libraries which have been prepared by point mutations or truncation and for screening cDNA libraries for gene products having a selected property are known in the prior art. These techniques can be adapted for rapidly screening the gene libraries which have been generated by combinatorial mutagenesis of variants of the invention. The most frequently employed techniques for screening large gene libraries which are subject to high-throughput analysis comprise cloning the gene library into replicable expression vectors, transforming the appropriate cells with the resulting vector library and expressing the combinatorial genes under conditions under which detection of the desired activity facilitates isolation of the vector which encodes the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which increases the frequency of functional mutants in the libraries, may be used in combination with the screening tests in order to identify variants (Arkin *et al.*, Proc. Acad. Natl. Sci., 1992, 89, 7811-7815; Delgrave *et al.*, Protein Engineering, 1993, 6, 327-331; in this case the term homolog is used interchangeably with the term variant).

Preferably, CAR is of enzyme class EC 1.2.1.30;

Preferably, PPTase is of enzyme class EC 2.7.8.7.

In another preferred embodiment, PPTase is a *Ec*PPTase or a *Cg*PPTase;

more preferably, is a PPTase of *E. coli*.

More preferably, CAR is of enzyme class EC 1.2.1.30 and PPTase is of enzyme class EC 2.7.8.7.

In another more preferred embodiment, CAR is of enzyme class EC 1.2.1.30 and PPTase is a *Ec*PPTase or a *Cg*PPTase; more preferably, is a PPTase of *E. coli*.

In an especial embodiment, CAR comprises each of the three amino acid sequences SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

each sequence is comprised with 80 to 100% identity, preferably with 90 to 100% identity, more preferably with 95 to 100% identity, even more preferably with 100% identity.

In a more especial embodiment, CAR comprises each of the three amino acid sequences SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

each sequence is comprised with 70 to 100% identity, preferably with 80 to 100% identity, more preferably with 90 to 100% identity, even more preferably with 95 to 100% identity, especially with 100% identity.

In an even more especial embodiment, CAR comprises each of the three amino acid sequences SEQ ID NO: 2, SEQ ID NO: 10 and SEQ ID NO: 6;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

each sequence is comprised with 70 to 100% identity, preferably with 80 to 100% identity, more preferably with 90 to 100% identity, even more preferably with 95 to 100% identity, especially with 100% identity.

In a particular embodiment, CAR comprises the CAR from *Nocardia* sp. strain NRRL 5646;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

the CAR from *Nocardia* sp. strain NRRL 5646 and each of the sequences SEQ ID NO: 12 and SEQ ID NO: 14 is comprised with 70 to 100% identity, preferably with 80 to 100% identity, more preferably with 90 to 100% identity, even more preferably with 95 to 100% identity, especially with 100% identity.

In a more particular embodiment, CAR comprises the sequence SEQ ID NO: 7;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

each sequence is comprised with 70 to 100% identity, preferably with 80 to 100% identity, more preferably with 90 to 100% identity, even more preferably with 95 to 100% identity, especially with 100% identity;

In an even more particular embodiment, CAR has the sequence SEQ ID NO: 7;

and PPTase has the sequence SEQ ID NO: 12 or has the sequence SEQ ID NO: 14;

each sequence with 70 to 100% identity, preferably with 80 to 100% identity, more preferably with 90 to 100% identity, even more preferably with 95 to 100% identity, especially with 100% identity.

In each of the above embodiments, the sequence SEQ ID NO: 12 is the preferred embodiment for PPTase.

Preferably, CAR and PPTase are used in the reaction

- in form of a cell free extract of a microorganism containing one of the enzymes or both, e.g. in form of a protein extract of a microorganism, that expressed one of the enzymes or both;
- in form of a microorganism, that expresses or expressed both enzymes, or in form of two mircoorganisms, one microorganism expressing the one enzyme and the other microorganism expressing the other enzyme; e.g. in form of a cell suspension of such microorganism;
- in form of parts of a microorganism, that expressed one of the enzymes or both, e.g. in form of cell debris of such microorganism; or
- as a combination of these embodiments.

The cell free extract containing the enzyme or both enzymes can be e.g. in form of a powdered protein composition, in form of a solution or suspension containing the enzyme or in form of a purified enzyme.

It is possible to use for the process of the invention growing cells. It is also possible to use resting or disrupted cells. Disrupted cells mean, for example, cells which have been made permeable by way of treatment with solvents for example, or cells which have been broken up by way of treatment with enzymes, by way of mechanical treatment (e.g. French press or ultrasonication) or by way of another method. The crude extracts obtained in this manner are advantageously suitable for the process of the invention. It is also possible to use purified or partially purified enzymes for the process. Immobilized microorganisms or enzymes which may advantageously be applied in the reaction are likewise suitable.

The microorganism, that expresses or expressed one of the enzymes or both, is also called host organism or host cell.

More preferably, CAR and PPTase are used in the reaction in form of a microorganism, that expresses both enzymes, or in form of two mircoorganisms, one microorganism expressing the one enzyme and the other microorganism expressing the other enzyme, or in form of a cell free extract containing the enzymes;

even more preferably in form of a microorganism, that expresses both enzymes, or in form of two mircoorganisms, one mircroorganism expressing the one enzyme and the other mircroorganism expressing the other enzyme.

It was also unexpected that the two enzymes CAR and PPTase can be maintained stably in one microorganism in form of two plasmids each carrying the gene of one of the eynzymes in spite of the fact that two identical origins of replications are present in this case.

It is possible to prepare recombinant microorganisms which are, for example, transformed with a vector carrying a gene or genes encoding for one of the enzymes or for both, and which are expressing one the enzymes or both. In this connection, familiar cloning and transfection methods known to the skilled worker, such as, for example, coprecipitation, protoplast fusion, electroporation, retroviral transfection and the like, are preferably used in order to cause said nucleic acids to be expressed in the particular expression system. Suitable systems are described, for example, in Current Protocols in Molecular Biology, F. Ausubel *et al.*, Eds., Wiley Interscience, New York 1997, or Sambrook *et al.*, Molecular Cloning: a Laboratory Manual. 2nd edition, Cold Spring 10 Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, or in "Molecular Cloning: a laboratory Manual, Third edition (3 volume set)", Cold Spring Harbor Laboratory Press, 2001.

A suitable microorganism used as host is in principle any prokaryotic or eukaryotic organism. Advantageously, microorganisms such as bacteria, fungi or yeasts are used as host organisms.

Preferred eukaryotic microorganisms are *Saccharomyces* or *Pichia*, more preferably *Saccharomyces cerevisiae* or *Pichia pastoris.*

Preferred prokaryotic microorganisms used as hosts are gram-positive or gram-negative bacteria, preferably selected from the group of families consisting of *Enterobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Streptomycetaceae* and *Nocardiaceae,* more preferably selected from the group consisting of *Escherichia, Bacillus, Lactobacillus, Pseudomonas, Streptomyces, Nocardia, Burkholderia, Salmonella, Agrobacterium* and *Rhodococcus*; even more preferably the microorganism is *Escherichia coli*. In addition, further advantageous bacteria can be found in the group of the alpha-proteobacteria, beta-proteobacteria or gamma-proteobacteria.

*E. coli* is used preferably in form of the strains selected from the group consisting of BL21, TG1, W311 0, DH1, XL 1-Blue and Origami, which are commercially available or can be obtained via the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Preferably, ECOLI-1 is *E. coli* BL21, more preferably *E. coli* BL21 (DE3).

The organisms used in the process of the invention are, depending on the host organism, grown or cultured in a manner known to the skilled worker. Microorganisms are usually grown in a liquid medium which comprises a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as iron salts, manganese salts, magnesium salts and, if appropriate, vitamins, at temperatures of between 0° C. and 100° C., preferably between 10° C and 60 °C, while being aerated. In this connection, the pH of the nutrient liquid may kept at a fixed value, i.e. it is not necessary but preferable to be regulated during cultivation. The cultivation may be carried out batch wise, semibatchwise or continuously. Nutrients may be introduced at the beginning of the fermentation or be fed in subsequently in a semicontinuous or continuous manner.

DOPAC may be added directly to the culture or, advantageously, after cultivation. The enzymes can be used according to the methods described in the examples or can be used for the reaction as a crude extract.

As cell culture system continuous or discontinuous culture such as batch culture or fed batch culture can be applied in culture tubes, shake flasks or bacterial fermenters. Host cells are usually cultured in conventional media as known in the art such as complex media like LB broth, "nutrient yeast broth medium", minimal media or a glycerol containing medium as described by Kortz *et al.*, J. Biotechnol., 1995, 39, 59-65 , or a mineral salt media as described by Kulla *et al.*, Arch. Microbiol., 1983, 135, 1-7.

LB broth and LB medium are used interchangeable.

Glucose can be added to the medium for cultivation.

Methods for induction, protein expression and harvesting of cells are known to the skilled person, and are described for example in "Molecular Cloning: a laboratory Manual, Third edition (3 volume set)", Cold Spring Harbor Laboratory Press, 2001.

Preferably, induction is done using IPTG.

The vector used in the invention is preferably an autonomously or self-replicating plasmid, a cosmid, a phage, a virus or a retrovirus. a wide variety of host/vector combinations may be employed in expressing the nucleic acid sequences encoding CAR and/or PPTase respectively. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and/or synthetic nucleic acid sequences. Suitable vectors include vectors with specific host range such as vectors specific for e. g. *E. coli* as well as vectors with broad-host-range such as vectors useful for Gram-negative bacteria. "Low-copy", "medium-copy" as well as "high copy" plasmids can be used.

Useful vectors for e.g. expression in *E. coli* are: pQE70, pQE60 und pQE-9 (QIAGEN, Inc.); pBluescriptVectors, PhagescriptVectors, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene Cloning Systems, Inc.); ptrc99a, pKK223-3, pKK233-3, pOR540, pRIT5 (Pharmacia Bio-tech, Inc.); pLG338, pACYC184, pBR322, pUC18, pUC19,pKC30, pRep4, pACYC177, pACYC184, pRSF1010 and pBW22 (Wilms *et al.*, Biotechnology and Bioengineering, 2001, 73, 95-103) or derivatives thereof such as plasmid pBW22-Fab-H or plasmid pAKL 14, pET Vectors (Novagene®) and pJexpress vectors (DNA2.0). Further useful plasmids are well known to the person skilled in the art and are described e.g. in "Cloning Vectors", Eds. Pouwels P. H. *et al.* Elsevier, Amsterdam-New York-Oxford, 1985.

Preferred vectors to be used in the method of the present inventions are autonomously or self-replicating plasmids, more preferred are vectors with specific host range such as vectors specific for e. g. *E. coli*.

Preferably, pJexpress vectors are used.

Preferably, when one of the enzymes CAR and PPTase or both are used as in form of a microorganism, that expresses both enzymes, or in form of two mircoorganisms, one mircroorganism expressing the one enzyme and the other mircroorganism expressing the other

enzyme, the respective microorganism is used in the reaction in form of its cells with an optical density of from 0.1 to 300, more preferably from 0.5 to 300, even more preferably from 1 to 300, especially from 5 to 300, more especially from 5 to 250, even more especially from 10 to 250, in particular from 10 to 200, more in particular from 50 to 200.

In another preferred embodiment, the amount of each of the enzymes CAR and PPTase in the reaction is from 0.001 to 100 mg/mL, more preferably from 0.005 to 100 mg/mL, even more preferably from 0.01 to 100 mg/mL, especially from 0.05 to 100 mg/mL, more especially from 0.1 to 30 mg/mL, even more especially from 0.5 to 30 mg/mL;

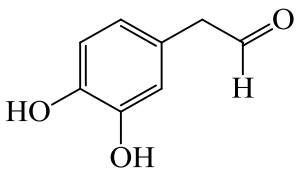
more preferably, the amount of each of the enzymes CAR and PPTase in the reaction, when used as in form of an isolated enzyme, is from 0.001 to 100 mg/mL, more preferably from 0.005 to 100 mg/mL, even more preferably from 0.01 to 100 mg/mL, especially from 0.05 to 100 mg/mL, more especially from 0.1 to 30 mg/mL, even more especially from 0.5 to 30 mg/mL.

In another preferred embodiment, the molar ratio of CAR : DOPAC in the reaction is from 1 : 50000 to 1 : 1; preferably from 1 : 10000 to 1 : 5, more preferably from 1 : 5000 to 1 : 20.

Preferably, the molar ratio of PPTase : CAR in the reaction is from 1 : 1000 to 1 : 1, more preferably from 1 : 500 to 1 : 2.

Preferably, DOPAC is used in the reaction in a concentration of from 0.1 to 100 mM, more preferably of from 1 to 100 mM, even more preferably of from 1 to 50 mM, especially of from 5 to 50 mM.

The reduction of DOPAC to 3-HT proceeds chemically in two steps: in the first reduction step the DOPAC is reduced to the corresponding aldehyde, the compound of formula (1),




(1)

in the second reduction step compound of formula (1) is reduced to 3-HT.

Preferably an enzyme SECREDSTEP is present in the reaction in addition to the two enzymes CAR and PPTase; enzyme SECREDSTEP is an enzyme which catalyzes the reduction of compound of formual (1) to 3-HT.

Enzyme SECREDSTEP can also be named in the literature as being an alcohol dehydrogenase, a aldo-keto reductase, a keto reductase, an aldehyde reductase or aldehyde hydrogenase.

Enzyme SECREDSTEP is preferably classified in the literature to be an enzyme that catalyzes the reaction, which enzymes of the enzyme class EC 1.1.1 catalyze.

According to IUBMB Enzyme Nomenclature and for the purpose of this invention, enzyme class EC 1.1.1 is assigned to enzymes called "EC 1 Oxidoreductases - EC 1.1 Acting on the CH-OH group of donors - EC 1.1.1 With NAD+ or $NADP^+$ as acceptor".

More preferably, enzyme SECREDSTEP is an enzyme classified in the literature to be an enzyme that catalyzes the reaction, which enzymes of enzyme classes EC 1.1.1.1 or EC 1.1.1.2 catalyze.

According to IUBMB Enzyme Nomenclature and for the purpose of this invention, enzyme class EC 1.1.1.1 is assigned to enzymes, that have the accepted name "alcohol dehydrogenase".

They catalyze the reaction:

"(1) a primary alcohol + $NAD^+$ = an aldehyde + NADH + $H^+$

(2) a secondary alcohol + $NAD^+$ = a ketone + NADH + $H^+$"

Other names are "aldehyde reductase; ADH; alcohol dehydrogenase (NAD); aliphatic alcohol dehydrogenase; ethanol dehydrogenase; NAD-dependent alcohol dehydrogenase; NAD-specific aromatic alcohol dehydrogenase; NADH-alcohol dehydrogenase; NADH-aldehyde dehydrogenase; primary alcohol dehydrogenase; yeast alcohol dehydrogenase".

Systematic name is "alcohol:$NAD^+$ oxidoreductase". The CAS registry number is 9031-72-5.

According to IUBMB Enzyme Nomenclature and for the purpose of this invention, enzyme class EC 1.1.1.2 is assigned to enzymes, that have the accepted name "alcohol dehydrogenase ($NADP^+$)".

They catalyze the reaction:

"an alcohol + $NADP^+$ = an aldehyde + NADPH + $H^+$"

Other names are "aldehyde reductase ($NADPH_2$); NADP-alcohol dehydrogenase; NADP-aldehyde reductase; NADP-dependent aldehyde reductase; NADPH-aldehyde reductase; NADPH-dependent aldehyde reductase; nonspecific succinic semialdehyde reductase; ALR 1; low-$K_m$ aldehyde reductase; high-$K_m$ aldehyde reductase; alcohol dehydrogenase (NADP)".

Systematic name is "alcohol:$NADP^+$ oxidoreductase". The CAS registry number is 9028-12-0.

It is possible to use enzyme SECREDSTEP in the reaction

- as an isolated enzyme; or
- to express enzyme SECREDSTEP separately from CAR and PPTase into a different microorganism, which is present in the reaction; or

- to express enzyme SECREDSTEP together with one of the enzymes CAR or PPTase or with both enzymes CAR and PPTase in the same microorganism, even on the same plasmid in the same microorganism; or
- enzyme SECREDSTEP is an endogenous enzyme of the micororganism that expresses or expressed CAR or PPTase or both.

Preferably, enzyme SECREDSTEP is an endogenous enzyme of the micororganism that expresses or expressed CAR or PPTase or both.

Preferably, when enzyme SECREDSTEP is used in the reaction in the form of a microorganism, that expresses enzyme SECREDSTEP, the respective microorganism is used in the reaction in the form of its cells with an optical density of from 0.1 to 300, more preferably from 0.5 to 300, even more preferably from 1 to 300, especially from 5 to 300, more especially from 5 to 250, even more especially from 10 to 250, in particular from 10 to 200, more in particular from 50 to 200.

In another preferred embodiment, the amount of enzyme SECREDSTEP in the reaction is from 0.001 to 100 mg/mL, more preferably from 0.005 to 100 mg/mL, even more preferably from 0.01 to 100 mg/mL, especially from 0.05 to 100 mg/mL, more escpecially from 0.1 to 30 mg/mL, even more especially from 0.5 to 30 mg/mL;

more preferably, the amount of enzyme SECREDSTEP in the reaction, when used as in form of an isolated enzyme, is from 0.001 to 100 mg/mL, more preferably from 0.005 to 100 mg/mL, even more preferably from 0.01 to 100 mg/mL, especially from 0.05 to 100 mg/mL, more especially from 0.1 to 30 mg/mL, even more especially from 0.5 to 30 mg/mL.

In another preferred embodiment, the molar ratio of enzyme SECREDSTEP : DOPAC in the reaction is from 1 : 50000 to 1 : 1; preferably from 1 : 10000 to 1 : 5.

Preferably, the reaction is done in an aqueous reaction medium.

Preferably, the reaction medium is a medium conventionally used for a cell culture.

More preferably, the reaction is done in the presence of a buffer (REAC).

Buffer (REAC) is a buffer commonly used in enzyme catalyzed reactions.

Preferably, buffer (REAC) is selected from the group consisting of phosphate buffer, HEPES (a buffer based on 4-2-hydroxyethyl-1-piperazineethanesulfonic acid), TES (a buffer based on 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid), MOPS (a buffer based on 3-(N-morpholino)propanesulfonic acid), PIPES (a buffer based on piperazine-N,N'-bis(2-ethanesulfonic acid), MES (a buffer based on 2-(N-morpholino)ethanesulfonic acid) and mixtures thereof;

more preferably, buffer (REAC) is phosphate buffer, MES or mixtures thereof.

A phosphate buffer is preferably a sodium or potassium phosphate buffer.

Even more, buffer (REAC) is sodium or potassium phosphate buffer or MES;

especially, buffer (REAC) is MES.

Preferably, the concentration of buffer (REAC) in the reaction mixture is from 0.005 to 0.5 M, more preferably from 0.02 to 0.2 M.

Even more preferably, the enzymes are used in the reaction in form of a microorganism, that expresses both enzymes, or in form of two mircoorganisms, one mircroorganism expressing the one enzyme and the other mircroorganism expressing the other enzyme;

and the reaction medium is buffer (REAC).

Preferably, the reaction is started in the presence of buffer (REAC) with a pH of from 5 to 8.5, more preferably of from 5.5 to 8.5, even more preferably of from 5.5 to 8.0, especially of from 5.5 to 7.6.

In another preferred embodiment, the reaction is done in the presence of buffer (REAC) with at a pH of from 5 to 8.5, more preferably of from 5.5 to 8.5, even more preferably of from 5.5 to 8.0, especially of from 5.5 to 7.6.

Preferably, the reaction is done in the presence of DTT. DTT is preferably added to the reaction mixture.

The amount of DTT is preferably from 0.01 to 20 mM, more preferably from 0.1 to 10 mM, even more preferably from 0.4 to 10 mM.

Preferably, the reaction is done in the presence of EDTA. EDTA is preferably added to the reaction mixture.

The amount of EDTA is preferably from 0.01 to 10 mM, more preferably from 0.1 to 5 mM, even more preferably from 0.4 to 5 mM.

Especially, the reaction is started in the presence of buffer (REAC) with a pH of from 5 to 8.5, and

the reaction is done in the presence of DTT, the amount of DTT is from 0.01 to 20 mM,

and

the reaction is done in the presence of EDTA, the amount of EDTA is from 0.01 to 10 mM;

more especially, the pH is from 5.5 to 8.5,

the amount of DTT is from 0.1 to 10 mM, and

the amount of EDTA is from 0.1 to 5 mM;

even more especially, the pH is from 5.5 to 8.0,

the amount of DTT is from 0.4 to 10 mM, and

the amount of EDTA is from 0.4 to 5 mM;

in particular, the pH is from 5.5 to 7.6,

the amount of DTT is from 0.4 to 10 mM, and

the amount of EDTA is from 0.4 to 5 mM.

Preferably, the reaction is done at a reaction temperature of from 4 to 45 °C, more preferably of from 10 to 45 °C, even more preferably of from 15 to 40 °C, especially of from 19 to 39 °C, more especially of from 20 to 38 °C.

Preferably, the reaction time is from 10 min to 96 h, more preferably from 20 min to 48 h.

Preferably, the reaction is done under aerobic conditions.

Preferably, the reaction medium is stirred, either by shaking the reaction tube or by a stirrer.

Preferably in case of shaking a small reaction vessel such as an eppendorf reaction tube, shaking is done at 500 to 1500 rpm, preferably at 750 to 1250 rpm.

Preferably in case of the use of a stirrer, stirring is done at 50 to 500 rpm.

Preferably, the reaction is done in the presence of a cofactor, the cofactor is selected from the group consisting of NADPH and NADH.

As cofactor either endogenous cofactor is used or the cofactor is added to the reaction mixture or both.

Preferably, when the cofactor is added, the cofactor is added to provide for a concentration of from 0.05 to 500 mM, more preferably of from 0.05 to 100 mM, even more preferably of from 0.1 to 50 mM.

In another preferred embodiment, when the cofactor is added, the cofactor is added to provide for a 2 to 10 fold molar amount based on the molar amount of DOPAC.

Preferably, endogenous cofactor is used.

The cofactor can be regenerated. For this purpose, preferably the reaction is

(i) done in the presence of an enzyme ENZREGENCOF, that regenerates the cofactor, and a respective substrate REGENSUB, that is used by ENZREGENCOF to regenerate the cofactor; or

(ii) the reaction is done using electrical current via redox electrodes for the regeneration of the cofactor; or

(iii) the reaction is done in the presence of a redox mediator for the regeneration of the cofactor; or

(iv) done in the presence of a substrate (SUBSCIT), substrate (SUBSCIT) is a substrate of the citric acid cycle or glucose.

More preferably, the reaction is

(i) done in the presence of an enzyme ENZREGENCOF, that regenerates the cofactor, and a respective substrate REGENSUB, that is used by ENZREGENCOF to regenerate the cofactor; or

(iv) done in the presence of a substrate (SUBSCIT).

The citric acid cycle is also known as tricarboxylic acid cycle (TCA cycle).

Preferably ENZREGENCOF is selected from the group consisting of glucose 1-dehydrogenase (GDH), formate dehydrogenase (FDH), phosphonate dehydrogenase, NAD(P) oxidase, alcohol dehydrogenase (ADH) and mixture thereof;

and REGENSUB is glucose, formate, phosphonate, hydrogen peroxide or an alcohol as substrate for the respective ENZREGENCOF;

more preferably, ENZREGENCOF is GDH with REGENSUB being glucose or ENZREGENCOF is FDH with REGENSUB being formate or ENZREGENCOF is a mixture of GDH and FDH with REGENSUB being glucose and formate;

even more preferably, ENZREGENCOF is GDH with REGENSUB being glucose.

ENZREGENCOF and REGENSUB are commercially available, e.g. from Sigma Aldrich, or ENZREGENCOF can be cloned according to known procedures from literature.

A redox mediator is preferably a viologen or a mixture of viologens. Viologens are bipyridinium derivatives of 4,4'-bipyridyl, preferred viologens are selected from the group consisting of methyl viologen with CAS 1910-42-5 and benzyl viologen with CAS 1102-19-8.

A substrate (SUBSCIT) is preferably selected from the group consisting of glucose citrate, cis-aconitate, D-isocitrate, alpha-ketoglutarate, succinate, fumarate, malate, oxaloacetate and mixtures thereof; more preferably glucose, citrate and mixtures thereof.

Preferably, substrate (SUBSCIT) is present in a concentration of from 0.001 to 0.5 M, more preferably of from 0.005 to 0.1 M.

In another preferred embodiment, substrate (SUBSCIT) is present in a 1 to 3 fold molar amount based on the molar amount of DOPAC.

Preferably, substrate (SUBSCIT) is added to the reaction mixture.

An alcohol as substrate for ADH can be any substrate that is a known substrate for AHD, preferably an alcohol as substrate for ADH is selected from the group consisting of $C_{2-8}$ alcohol; more preferably ethanol, propanol or butanol.

For the purpose of this invention, GDH is preferably an enzyme of the enzyme class EC 1.1.1.47.

According to IUBMB Enzyme Nomenclature, enzyme class EC 1.1.1.47 is assigned to enzymes that have the accepted name "glucose 1-dehydrogenase".

They catalyze the reaction:

"beta-D-glucose + $NAD(P)^+$ = D-glucono-1,5-lactone + NAD(P)H + $H^+$"

Other names are "D-glucose dehydrogenase (NAD(P)); hexose phosphate dehydrogenase".

Systematic name is "beta-D-glucose: $NAD(P)^+$ 1-oxidoreductase". The CAS registry number is 9028-53-9.

For the purpose of this invention, FDH is preferably an enzyme of the enzyme class EC 1.2.1.2.

According to IUBMB Enzyme Nomenclature, enzyme class EC 1.2.1.2 is assigned to enzymes, that have the accepted name "formate dehydrogenase".

They catalyze the reaction:

"formate + $NAD^+$ = $CO_2$ + NADH"

Other names are "formate-NAD oxidoreductase; FDH I; FDH II; N-FDH; formic hydrogen-lyase; formate hydrogenlyase; hydrogenlyase; NAD-linked formate dehydrogenase; NAD-dependent formate dehydrogenase; formate dehydrogenase (NAD); NAD-formate dehydrogenase; formate benzyl-viologenoxidoreductase; formic acid dehydrogenase".

Systematic name is "formate: $NAD^+$ oxidoreductase". The CAS registry number is 9028-85-7.

For the purpose of this invention, phosphonate dehydrogenase is preferably an enzyme of the enzyme class EC 1.20.1.1.

According to IUBMB Enzyme Nomenclature, enzyme class EC 1.20.1.1 is assigned to enzymes, that have the accepted name "phosphonate dehydrogenase".

They catalyze the reaction:

"phosphonate + $NAD^+$ + $H_2O$ = phosphate + NADH + $H^+$"

Other names are "NAD: phosphite oxidoreductase; phosphite dehydrogenase".

Systematic name is "phosphonate: $NAD^+$ oxidoreductase". The CAS registry number is 9031-35-0.

For the purpose of this invention, NAD(P) oxidase is preferably an enzyme of the enzyme class EC 1.6.3.1.

According to IUBMB Enzyme Nomenclature, enzyme class EC 1.6.3.1 is assigned to enzymes, that have the accepted name "NAD(P)H oxidase".

They catalyze the reaction:

"$NAD(P)H + H^+ + O_2 = NAD(P)^+ + H_2O_2$"

Other names are "THOX2; ThOX; dual oxidase; p138tox; thyroid NADPH oxidase; thyroid oxidase; thyroid oxidase 2; NADPH oxidase".

Systematic name is "NAD(P)H: oxygenoxidoreductase". The CAS registry number is 77106-92-4.

For the purpose of this invention, ADH is preferably an enzyme of the enzyme class EC 1.1.1.1 or of the enzyme class EC 1.1.1.2.

Preferably, the amount of ENZREGENCOF is from 0.001 to 100 mg/mL, more preferably from 0.005 to 100 mg/mL, even more preferably from 0.005 to 30 mg/mL, especially from 0.005 to 20 mg/mL; more especially from 0.005 to 15 mg/mL.

In another preferred embodiment, the molar ratio of ENZREGENCOF : DOPAC is from 1 : 50000 to 1 : 1; preferably from 1 : 10000 to 1 : 1.

Since the enzymes CAR, PPTase, optionally enzyme ENZREGENCOF and optionally enzyme SECREDSTEP can be used in the reaction either expressed in one, in two, in three or even in four microorganisms, the total optical density of the combined microorganisms in the reaction is preferably of from 0.1 to 300, more preferably from 0.5 to 300, even more preferably from 1 to 300, especially from 5 to 300, more especially from 5 to 250, even more especially from 10 to 250, in particular from 10 to 200, more in particular from 50 to 200.

Preferably, the molar amount of REGENSUB is from 1 to 10 fold, more preferably from 1 to 6 fold, based on the molar amount of DOPAC.

It is possible to use ENZREGENCOF as isolated enzyme, or to clone ENZREGENCOF separately from CAR and PPTase into a different microorganism, which then is present in the reaction, or to clone ENZREGENCOF together with one of the enzymes CAR or PPTase or with both enzymes CAR and PPTase in the same microorganism, even on the same plasmid in the same microorganism.

Preferably, the reaction is done in the presence of a kation KAT, KAT is selected from the group consisting of $Mg^{2+}$, $Zn^{2+}$ and mixtures thereof.
Preferably, KAT is present in form of a sulfate, hydrogen sulfate, chloride or acetate of KAT.
Preferably, KAT is $Mg^{2+}$;
more preferably, KAT is present in form of a sulfate, hydrogen sulfate, chloride or acetate of $Mg^{2+}$ or as a mixture thereof;
even more preferably in form of $MgSO_4$ or $MgCl_2$;
especially in form of $MgCl_2$.

Preferably, KAT as added to the reaction mixture.

Preferably, KAT is used in a concentration of from 0.01 to 50 mM, more preferably of from 0.1 to 30 mM, even more preferably 0.5 to 30 mM, especially 0.5 to 20 mM.

In particular, the reaction is started in the presence of buffer (REAC) with a pH of from 5 to 8.5, and

the reaction is done in the presence of DTT, the amount of DTT is from 0.01 to 20 mM, and

the reaction is done in the presence of EDTA, the amount of EDTA is from 0.01 to 10 mM, and

KAT is used in a concentration of from 0.01 to 50 mM;

more in particular, the pH is from 5.5 to 8.5,

the amount of DTT is from 0.1 to 10 mM, and

the amount of EDTA is from 0.1 to 5 mM, and

KAT is used in a concentration of from 0.01 to 50 mM;

even more in particular, the pH is from 5.5 to 8.0,

the amount of DTT is from 0.4 to 10 mM, and

the amount of EDTA is from 0.4 to 5 mM, and

KAT is used in a concentration of from 0.1 to 30 mM;

specifically, the pH is from 5.5 to 7.6,

the amount of DTT is from 0.4 to 10 mM, and

the amount of EDTA is from 0.4 to 5 mM, and

KAT is used in a concentration of from 0.5 to 30 mM;

with KAT as defined above, also with any of its preferred embodiments.

Preferably, if the reaction is done with resting cells, which are frozen and are thawed for the reaction, these cells should be frozen for a time period of up to one month. To have the cells for a longer period of time in the frozen state might reduce their activity. Generally, this was observed in the disclosed experiments.

The reaction can be monitored, e.g. by HPLC, GC or by NMR.

After the reaction, 3-HT can be isolated from the reaction mixture by standard methods known to the skilled person. Preferably, the cells are separated by centrifugation or filtration. The product is preferably isolated by extraction with a solvent, preferably after centrifugation or filtration, from the mother liquor, and can be purified by flash column chromatography or crystallization or both.

Preferably, extraction is done with a solvent EXTR selected from the group consisting of toluene, dichloromethane, cyclohexane, methylcyclohexane, ethyl acetate and methyl tert-butyl ether, preferably ethyl acetate.

Flash column chromatography is preferably done using a column packed with silicagel G 60, the solvent system for flash chromatography is preferably methyl tert-butyl ether : n-heptane 1:1 v/v.

Crystallization is preferably done from a solvent CRYST selected from the group consisting of petroleum : ether, 3.5 : 1 v/v; the ether is preferably methyl tert-butyl ether.

The foregoing description will be more fully understood with reference to the following Examples. Such examples, are, however, exemplary of methods of practicing the present invention and are not intended to limit the scope of the invention. Especially techniques of genetic engineering, such as cloning, transformation and sequencing, also sequence verification after cloning, can be carried out in accordance with procedures described in "Molecular Cloning: a laboratory Manual, Third edition (3 volume set)", Cold Spring Harbor Laboratory Press, 2001.

### 7.4.3 Examples

**Materials, equipment, sources, further abbreviations**

Amp Ampicillin, Sigma Aldrich, www.sigmaaldrich.com

ACN Acetonitrile, J. T. Baker, Aventor Performance Materials B.V., The Netherlands.

ATP Adenosine-5'-triphosphate, Roche, Basel, Switzerland, www.roche.com

Bacto Yeast Extract Becton, Dickinson and Company, NJ USA 07417, www.bd.com

Buffer A 50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol, pH 7.5 adjusted with HCl

Buffer B 50 mM MES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol, pH 6.0 adjusted with NaOH

Buffer C 50 mM MES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, pH 6.0 adjusted with NaOH

Buffer D 50 mM MES, pH 6.0 adjusted with NaOH

CD cell debris – debris of cells and possibly remaining unbroken cells which result from sonication and ultracentrifugation

Centrifuges: Small: (e.g. for Eppendorf tubes) Centrifuge 5415R (Eppendorf)

Medium: (e.g. for Falcon tubes) Centrifuge 5810R (Eppendorf)

Big: (e.g. for volumes of more than 50 mL) Avanti centrifuge J-20 (Beckman Coulter, www.beckmancoulter.com)

Ultracentrifuge: Optima LE80K (Beckman Coulter, www.beckmancoulter.com)

Centrifugation parameter can also be specified with the g value (gravitational constant)

CFE cell free extract

Chemically competent cells *E. coli*BL21 (DE3) Gold Biocompare, South San Francisco, CA 94080, USA, www.biocompare.com

CoA Coenzyme a trilithium salt, Sigma Aldrich, www.sigmaaldrich.com

DOPAC Sigma-Aldrich, www.sigmaaldrich.com

DTT Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com

EDTA Carl ROTH,76185 Karlsruhe, Germany, www.carlroth.com

Electrocompetent cells *E. coli*BL21 (DE3) Gold Agilent Technologies, Inc., CA 95051 United States, www.genomics.agilent.com

Fermentas FERMENTAS GMBH, St. Leon-Rot, Germany, a subsidiary of Thermo Fisher Scientific Inc., www.fermentas.com

Finnzymes part of Thermo Scientific Molecular Biology, www.thermoscientificbio.com

Glucose monohydrate Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com

Glycerol Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com

3-HT can be purchased from Sigma-Aldrich, www.sigmaaldrich.com

Invitrogen Invitrogen Corporation, www.invitrogen.com, a subsidiary of Life Technologies Corporation, www.lifetechnologies.com

IPTG isopropyl beta-D-1-thiogalactopyranoside, Biosynth, 9422 Staad, Switzerland, www.biosynth.com

pJexpress IP-Free™ bacterial expression vectors of DNA2.0, information reproduced in this specification is provided on the internet homepage of DNA2.0 and is reproduced with permission of DNA2.0

pJexpress 401 plasmid of DNA2.0 with kanamycin resistance maker and T5 promoter, information is provided on the internet homepage of DNA2.0

pJexpress 404 plasmid of DNA2.0 with ampicillin resistance maker and T5 promoter, information is provided on the internet homepage of DNA2.0

Kan Kanamycin, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com

$KH_2PO_4$ Potassium dihydrogen phosphate, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com

$K_2HPO_4$ di-Potassium hydrogen phosphate, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com

| | |
|---|---|
| KPi | Phosphate buffer, containing different ratio of equalmolar $KH_2PO_4$ and $K_2HPO_4$, depending on desired pH, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| LB-plates | Luria broth medium (also called LB-Lennox-Agar) (20g/L) containing agarose (15g/L) and optionally the appropriate antibiotic, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| LB medium | Luria broth medium (also called LB-Lennox) (20g/L: 10g/L Tryptone, 5g/L Yeast extract, 5 g/L NaCl) , Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| LGC Genomics | LGC Genomics GmbH, Berlin, Germany, www.lgcgroup.com |
| MES | 2-(N-morpholino)ethanesulfonic acid, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| MES buffer | 50 mM MES, pH adjusted with 1 M NaOH |
| $MgCl_2$ | Magnesium chloride, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| $MgSO_4$ | Magnesium sulfate, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| MWD | multi wavelength detector |
| $NAD^+$ | Nicotinamide adenine dinucleotide, oxidized form, Roche Diagnostics, www.roche.com |
| NADH | Nicotinamide adenine dinucleotide, Roche Diagnostics, www.roche.com |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate, oxidized form, Roche Diagnostics, www.roche.com |
| NADPH | Nicotinamide adenine dinucleotide phosphate, Roche Diagnostics, www.roche.com |
| NaOH | Sodium hydroxide, Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| PageRuler™ | Prestained Protein Ladder Fermentas#SM0671, published by Thermo Scientific under "CERTIFICATE OF ANALYSIS PageRuler™ Prestained Protein Ladder#SM0671" in the internet at www.fermentas.com |
| SOC medium | Super Optimal broth with Catabolite repression medium: 2% w/v Tryptone, 0.5% w/v Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ , 10 mM $MgSO_4$, 20 mM Glucose |
| Tris | tris-(hydroxymethyl)-aminomethane (IUPAC name: 2-Amino-2-hydroxymethyl-propane-1,3-diol), Carl ROTH, 76185 Karlsruhe, Germany, www.carlroth.com |
| Tryptone | Tryptone is the assortment of peptides formed by the digestion of casein by the protease trypsin, supplier: Oxoid Limited, Hampshire, United Kingdom, www.oxoid.com, a subsidiary of Thermo Fisher Scientific, www.thermofisher.com, |

U 1 enzyme unit (U) = 1 µmol $min^{-1}$, that is 1 U converts 1 µmol of substrate per minute

## Methods

**OD** Optical Density measured at 600 nm in cuvettes with a pathlength of 1 cm

**HPLC Method A**

HPLC Agilent Technologies 1200 Series equipped with G1379B degasser,G1312B Binary Pump SL, G1367C HiP-ALS SL Autosampler including well plate handler, G1314C VWD SL UV detector, G1316B TCC SL column oven and G1956B MSD Mass selective detector.

Injection volume 10µL

Column Chromolith Performance RP-18 end capped 100-4.6 operated at 30°C

Flow 1.2 mL/min, split flow to 0.8 mL/min between UV and MS detector

Solvent 95% formic acid 0.1%; 5% ACN

Detection either MS: e.g. Agilent G1956B, equipped with Atmospheric pressure electrospray ionization, positive mode (MS) recording for scan (90 to 450 m/z) and SIMs (155 m/z and 153 m/z for alcohol and aldehyde, resp.) and negative mode (for acid)

and/or UV: e.g. Agilent 1100 equipped with MWD recording 230 or 254 nm

Evaluation: Results are given in the examples in concentration and/or as% value. To determine the concentration, a calibration was done with known concentration of the respective substance to be detected; the % value is the analytical yield of product (product is 3-HT if not otherwise stated) based on the molar amount of substrate used; if not otherwise stated.

In some experiments more than the theoretical possible yield of 3-HT was detected and is reported in the results. This stems from the refilling after reaction of the Eppendorf tubes to a volume of 500 µL, which was done visually using a reference mark for the volume on the Eppendorf tubes, and introduced a respective inaccuracy.

**Protocol of DNA2.0 delivered with the plasmids for retrieval of plasmid DNA**

Instructions for Use

Accessing plasmid DNA from GFC Filter

- Remove filter from plastic bag and place on a sterile and clean surface.
- Add 100 µL of 10 mM Tris-HCl, pH 7.5 directly to the center of the filter.
- Incubate at room temperature for 2 min.

- Puncture the bottom of a 0.6 mL tube using a syringe.
- Place filter in the 0.6 mL tube and place the 0.6 mL tube in a 1.5 mL tube.
- Place the 1.5 mL tube (now containing punctured 0.6 mL tube with filter) in tabletop centrifuge.
- Spin 1 min at full speed. The DNA containing liquid will transfer from the filter in the 0.6 mL tube to the 1.5 mL tube.
- Discard the 0.6 mL tube with filter. The 1.5 mL tube now contains ca. 90 µL buffer and DNA.
- Carefully remove supernatant. There may be a small pellet consisting of filter debris. This pellet does not contain any of the DNA. The supernatant should contain approximately 2 µg plasmid DNA (ca. 20 ng/µL).

The isolated DNA can subsequently be transformed, cut with restriction enzymes, or sequenced without further purification.

**NuPAGE® electrophoresis (Invitrogen)**

NuPAGE® electrophoresis was performed to check the overexpression of the target proteins as follows:

**Standard:** 5 µl PageRuler™Prestained Protein Ladder (Fermentas)

**Samples:** 15 µl of cell free extract (CFE) or cell debris (CD) with protein concentration 2 mg/mL + 5 µl of NuPAGE® LDS Loading Buffer (4X) (Invitrogen), boiled at 99°C for 5 min on Thermomixer (Eppendorf), spun on Centrifuge 5415R (Eppendorf), loaded on the gel.

**Gel:** 4 to 12 % NuPAGE® Bis-Tris Gel (Invitrogen)

**Running Buffer:** NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X)

**Power Supplier:** PowerEase® 500 Power Supply (Invitrogen)

**Electrophoresis cell:** XCellSureLock® Mini-Cell

Electrophoresis was run for 45 min at 200 V.

**Staining:** The gel was washed 3 times for 5min with distilled water, stained for 1 h with SimplyBlue™ SafeStain (Invitrogen) and destained overnight in distilled water under shaking on PMR-30 Platform Rocker (Grant Bio, subsidiary of Keison, www.keison.co.uk). After that the gel was scanned.

**Purification Protocol of GeneJET™ Plasmid Miniprep Kit of Fermentas**

published by Thermo Scientific under "PRODUCTINFORMATION Thermo Scientific GeneJET Plasmid Miniprep Kit#K0502, #K0503"in the internet at www.thermoscientific.com/fermentas, © 2011 Thermo Fisher Scientific Inc.

**Sonication**

Sonication is done as follows, if not otherwise stated:

The cell pellets were dispersed in buffer. Typically, per gram of wet cell paste, 10 mL of buffer was used. The dispersion was placed into an aluminium beaker that was placed on a stirred water-ice bath. a Branson Sonifier® 250 analog ultrasonic cell disruptor equipped with a 1/2" tapped Disruptor horn was used for 6 min set to 80% duty cycle and to output control 8.

**Example 1 - Strains and cultivation**

The gene with sequence SEQ ID NO: 9 and the gene with sequence SEQ ID NO: 11 were ordered from DNA2.0 and were delivered in form of plasmid pJexpress-404-9-CAR with SEQ ID NO: 15, carrying the gene with SEQ ID NO: 9 for CAR, and of plasmid pJexpress-401-11-EcPPTase with SEQ ID NO: 16, carrying the gene for PPTase with SEQ ID NO: 11, on GFC filters.

FIG 1 shows pJexpress-404-9-CAR.

Legend of FIG 1:

| | |
|---|---|
| pUC ori | origin of replication of the plasmid pUC (p short for "plasmid", "UC" short for "University of California"), which is one of the most common origins of replication that is used in plasmids for genetic engineering |
| rpn txn terminator | terminator of the rpn txn gene |
| Primer 1 to 6 | as defined under List of Sequences |
| bla txn terminator | terminator of the bla txn gene |
| T5 promoter | T5 promoter sequence was described in Gentz *et al.*, J. Bacteriol. 1985, 164, 70-77. Silencing of the promoter prior to IPTG induction is achieved using symmetrical lac operators (Sadler *et al.*, Proc. Natl. Acad. Sci. USA 1983, 80, 6785-6789) spaced around the promoter to maximize cooperativity (Oehler *et al.*, EMBO J., 1994, 13, 3348-3355). This operator pair ensures significantly tighter repression than regular lac operators. Overlapping T5 promoter/lac operator has been described (Lanzer *et al.*, Proc. Natl. Acad. Sci. USA, 1988, 85, 8973-8977) |
| CAR of SEQ ID NO: 9 | gene with SEQ ID NO: 9 for CAR |
| rrnB1 B2 T1 txn terminator | terminator of the rrnB1 B2 T1 txn gene |
| amp | gene for ampicillin resistance |
| lacI | lacI gene, which expresses the repressor protein that binds to the lac operator |

FIG 2 shows pJexpress-401-11-EcPPTase.

Legend of FIG 2:

| | |
|---|---|
| pUC ori | origin of replication of the plasmid pUC (p short for "plasmid", "UC" short for "University of California"), which is one of the most common origin of replication that is used in plasmids for genetic engineering |
| rpn txn terminator | terminator of the rpn txn gene |
| Primer 1 and 2 | as defined under List of Sequences |
| bla txn terminator | terminator of the bla txn gene |
| T5 promoter | T5 promoter sequence was described in Gentz *et al.*, J. Bacteriol 1985, 164, 70ff. Silencing of the promoter prior to IPTG induction is achieved using symmetrical lac operators (Sadler et al, Proc. Natl. Acad. Sci. USA 1983, 80, 6785-6789) spaced around the promoter to maximize cooperativity (Oehler et al, EMBO J 1994, 13, 3348-3355). This operator pair ensures significantly tighter repression than regular lac operators. Overlapping T5 promoter/lac operator has been described (Lanzer et al, Proc. Natl. Acad. Sci. USA, 1988, 85, 8973-8977) |
| *Ec*PPTase of SEQ ID NO: 11 | gene with SEQ ID NO: 11 for EcPPTase |
| rrnB1 B2 T1 txn terminator | terminator of the rrnB1 B2 T1 txn gene |
| kan | gene for kanamycin resistance |
| lacI | lacI gene, which expresses the repressor protein that binds to the lac operator |

Plasmid DNA was retrieved according to the protocol of DNA2.0 delivered with the plasmids (see above under Methods).

60 µL of chemically competent cells *E. coli* BL21 (DE3) Gold were transformed with the plasmids separately, using 100 ng of pJexpress-404-9-CAR to give strain CE1 and 75 ng of pJexpress-401-11-EcPPTase to give strain PE1 respectively. The transformation was done as follows: The two mixtures of cells and plasmid were incubated on ice for 30 min, heat shocked for 30 s at 42°C and cooled down on ice for 2 min. 250 µL of pre-warmed SOC medium was added, incubated 1 h at 37°C.

The strains were selected as follows:

100 µL from each transformation was streaked on LB-plates, supplemented with 100 µg/mL of Amp or with 50 µg/mL Kan respectively, and incubated overnight at 37°C.

Plasmid DNA was isolated from the transformed cells CE1 and PE1 according to the Purification Protocol of GeneJET™ Plasmid Miniprep Kit of Fermentas.

Plasmid DNA isolated from PE1 and CE1 was sequenced by LGC genomics using Primers 1 to 6 for SEQ ID NO: 9 and Primers 1 and 2 for SEQ ID NO: 11 and confirmed SEQ ID NO: 9 and SEQ ID NO: 11 respectively.

Primer 1 is called pTF5 by DNA2.0, Primer 2 is called pTR by DNA2.0.

Strains CPE1 and CPE2 were obtained by simultaneous transformation with two plasmids of electrocompetent cells as follows:

For transformation, ice cooled electroporation cuvettes 2 mm (Cell Projects Ltd, www.cellprojects.com) were filled with 80 µL of electrocompetent cells *E. coli* BL21 (DE3) Gold for CPE1 and with 1.13 µg of pJexpress-404-9-CAR and 0.11 µg of pJexpress-401-11-EcPPTase; and with 80 µL of electrocompetent cells *E. coli* BL21 (DE3) Gold for CPE2 and with 0.3 µg of pJexpress-404-9-CAR and 0.03 µg of pEHISTEV-14-CgPPTase prepared according to example 9 respectively. After 2 min incubation on ice, electroporation cuvettes were placed in a MicroPulserElectroporator411BR (Bio-Rad, www.bio-rad.com) and electroporation with following conditions 25 mFD, 200 W, and 2.5 kV (time constant ca. 5 msec) was started. Afterwards, the cells were transferred to a 1.5 mL tube, 600 µL of SOC medium was added and the cells were incubated on a Thermomixer comfort (Eppendorf) at 37°C and 600 rpm for 1 h.

The strains were selected as follows:

Next, 100 µL of cell suspension was streaked on LB-plate supplemented with 100 µg/mL Amp and 50 µg/mL Kan and incubated overnight at 37°C.

Preparation of glycerol stock samples:

20 mL of LB medium containing 100 mg/L Amp and/or 50 mg/L Kan respectively was inoculated with a single colony of one the strains CE1, PE1, CPE1 and CPE2 respectively. Cultivation was carried out overnight at 37°C and 120 rpm. Samples of 500 µL were mixed with 250 µL LB medium and 250 µL aqueous glycerol 50% v/v and shock-frozen in liquid nitrogen and stored at -80°C to provide so called glycerol stock samples.

The strains were cultivated as follows:

20 mL of LB medium containing 100 mg/L Amp and/or 50 mg/L Kan respectively was inoculated with 5 µL of a glycerol stock sample of one the strains CE1, PE1, CPE1 and CPE2 respectively. Cultivation was carried out overnight at 37°C and 120 rpm. Subsequently, 500 mL LB medium, containing 100 mg/L Amp and/or 50 mg/L Kan respectively, in sterile 2L baffled flask were inoculated with pre-culture in such an amount in order to obtain an OD of ca. 0.1. Incubation for 1.5 to 2 h at 37°C and 120 rpm delivered cultures with an OD between 0.4 and 0.6, which were induced with 1 mM IPTG (0.5mL of 1M aqueous solution). The protein expression phase proceeded for 4 h at 37°C and 120 rpm. Subsequently, cells were harvested by centrifugation at 2831 g, 4°C for 10 min. The cell pellet was washed twice with Buffer A, and centrifuged again at 2831 g, 4°C for 10

min. The supernatant was removed and the cell pellet was dispersed in 50% glycerol and shock-frozen as 1 mL aliquots in liquid nitrogen. These resting cell samples were stored at -20 °C.

Protein expression was visualized for CE1, PE1 and CPE1 with NuPage® electrophoresis. FIG 3 shows overexpression of CAR, expected molecular masses are 128 kDa for CAR and 23 kDa for EcPPTase.

The NuPage® electrophoresis was done with a concentration of ca. 1 mg/mL of total protein and the amount of CAR was estimated by visual inspection of the strength of the bands. This results in a approximate concentration of CAR of ca. 8 µM in the enzymatic reduction reactions described in the following examples.

FIG 3 shows, that whereas the over expression of CAR enzyme is evident in CFE in lane 2, EcPPTase is not visible in overexpressed amounts. However, the presence of sufficient EcPPTase is evident because CPE1 cells are active for carboxylate reduction whereas the CE1 strain is inactive.

**Legend of FIG 3:**

Lane No

1 PageRuler™ Prestained Protein Ladder

2 CFE of CPE1

3 CD of CPE1

**Example 2**

With the resting cell samples, prepared according to Example 1 and stored at -20°C, several reaction conditions were tested, details are given in table 2. For this purpose, the 1 mL aliquots were thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with reaction buffer, the reaction buffer was 50 mM KPi buffer of pH 7.5. After the second centrifugation and buffer removal, in case of addition of CoA, the cell pellets were dispersed in the reaction buffer and CoA was added and the cells were preincubated for 1 h at 28 °C and at 1000 rpm prior to addition of the other ingredients . In case of no addition of CoA, the cell pellets were dispersed in the reaction buffer and the other ingredients were added without said preincubation.

The other ingredients were added according to table 2 to have the stated concentrations in the reaction mixture.

Reactions were done with the following parameters:

- with whole cells of strain CPE1,
- addition of $MgCl_2$ for a concentration of 20 mM,
- addition of DOPAC for a concentration of 20 mM,
- reaction buffer was used to adjust the final volume to 500 µL
- the pH after addition of all ingredients was not controlled,
- incubation over night in Eppendorf tubes at 28 °C with open or closed lids.

After incubation, the reaction mixtures were filtered through syringe filters (size 0.2 µm, nylon) and analyzed by HPLC Method A.

Results are summarized in Table 2.

| **Table 2** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Example** | **CoA [mM]** | **ATP [mM]** | **NADPH [mM]** | **Glucose [mM]** | **Toluene [mM]** | **Lid** | **3-HT [mM]** |
| 2-1 | 1 | 40 | 40 | --- | 38 | closed | 1.2 |
| 2-2 | 1 | --- | --- | 200 | 38 | closed | 0.08 |
| 2-3 | --- | --- | 40 | --- | 38 | closed | 0.85 |
| 2-4 | 1 | --- | 40 | --- | 38 | closed | 0.79 |
| 2-5 | 1 | --- | 40 | 200 | 38 | closed | 1.2 |
| 2-6 | 1 | --- | 40 | --- | 38 | open | 7.3 |
| 2-7 | 1 | --- | 40 | 200 | 38 | open | 13.8 |
| 2-8 | 1 | 40 | 40 | --- | 38 | open | 16.2 |
| 2-9 | --- | --- | 40 | --- | 38 | open | 14.3 |
| 2-10 | --- | --- | 40 | 200 | 38 | open | 4.02 |
| 2-11 | --- | --- | 40 | --- | --- | open | 11.4 |

**Example 3 - various reaction parameters**

The effect of reaction mixture composition was studied under the following conditions:

CPE1 resting cell sample aliquots, prepared according to Example 1, were thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with buffer B. The cell pellets were dispersed in 50 mM MES buffer pH 6.0 containing variations of components as specified in Table3. 0.5 M NADPH in water and 100 mM of DOPAC in water were added to give final concentrations of 20 mM and 10 mM respectively, in a total volume of 500 µL. The thus prepared samples in Eppendorf vessels were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm for 4 h with open lid. The samples were refilled with water to 500 µL, centrifuged for 10 min at 28 °C and the supernatants were filtered through plate filters (0.2 µm, polypropylene). The filtrates were analyzed with Method A.

"Relative %" means that the % value of Ex 3-1 was set to 100% and all other % values were normalized to this value.

"Relative DOPAC and 3-HT recovery [%]" means the value of the sum of substrate and product concentration of Ex 3-16 was set to 100% and all other values were normalized to this value

| Table 3 | | | | | | |
|---|---|---|---|---|---|---|
| **Example** | **$MgCl_2$ [mM]** | **DTT [mM]** | **EDTA [mM]** | **Glycerol [% v/v]** | **Relative %** | **Relative DOPAC and 3-HT recovery [%]** |
| 3-1 | 0 | 0 | 0 | 0 | 100 | 76 |
| 3-2 | 10 | 0 | 0 | 0 | 148 | 48 |
| 3-3 | 10 | 0 | 1 | 0 | 80 | 103 |
| 3-4 | 10 | 1 | 0 | 0 | 69 | 97 |
| 3-5 | 0 | 0 | 1 | 0 | 45 | 102 |
| 3-6 | 0 | 1 | 0 | 0 | 85 | 45 |
| 3-7 | 0 | 1 | 1 | 0 | 68 | 98 |
| 3-8 | 10 | 1 | 1 | 0 | 89 | 95 |
| 3-9 | 0 | 0 | 0 | 10 | 68 | 100 |
| 3-10 | 10 | 0 | 0 | 10 | 77 | 107 |
| 3-11 | 10 | 0 | 1 | 10 | 26 | 109 |
| 3-12 | 10 | 1 | 0 | 10 | 43 | 106 |
| 3-13 | 0 | 0 | 1 | 10 | 77 | 108 |
| 3-14 | 0 | 1 | 0 | 10 | 74 | 105 |
| 3-15 | 0 | 1 | 1 | 10 | 68 | 99 |
| 3-16 | 10 | 1 | 1 | 10 | 51 | 100 |

**Example 4– temperature of reaction**

The reaction temperature was studied under the following conditions: CPE1 resting cell sample aliquots, which can be prepared according to Example 1, were thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with buffer B. The cell pellets were dispersed in buffer B, and 0.5 M NADPH in water and 100 mM of DOPAC in water were added to give final concentrations of 20 mM and 10 mM respectively, in a total volume of 500 µL. The samples were incubated in five separate Eppendorf Thermomixers at different temperatures at 1000 rpm with open lid as outlined in table 4. After 16 h, the samples were refilled with water to 500 µL, centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm, polypropylene). The filtrates were analyzed with Method A. Table 4 shows the influence of the reaction temperature on the reaction.

"Relative %" means that the % value of Ex 4-3 was set to 100% and all other % values were normalized to this value.

| Table 4 | | |
|---|---|---|
| **Example** | **Temperature [°C]** | **Relative [%]** |
| 4-1 | 20 | 17 |
| 4-2 | 24 | 61 |
| 4-3 | 28 | 100 |
| 4-4 | 30 | 79 |
| 4-5 | 34 | 76 |

**Example 5 - time of reaction**

The influence of the reaction time was studied under the following conditions: CPE1 resting cell samples were prepared according to Example 1.The tested reaction times are given in table 5.

For the reaction times of 16 h to 24 h, CPE1 fresh cells were used, which were prepared according to example 1, wherein the cells were harvested as described in example 1 by centrifugation at 2831 g at 4°C for 10 min after the protein expression phase, resulting in a cell pellet of fresh cells. These fresh cell pellets were washed with buffer B and then dispersed in buffer B.

For the reaction times of 30 min to 8 h, CPE1 resting cell sample aliquots, prepared according to Example 1, were thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with buffer B. These cell pellets were dispersed in buffer B.

To any of these cell pellets dispersed in buffer B, 0.5 M NADPH in water and 100 mM of DOPAC in water were added to give final concentrations of 20 mM and 10 mM respectively, in a total volume of 500 µL.

The thus prepared samples in Eppendorf vessels were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm with open lid. At the reaction times indicated in Table5, the samples were refilled with water to 500 µL, centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm polypropylene). The filtrates were analyzed with Method A. Table 5 shows the influence of the reaction time of the reaction.

| **Table 5** | | | |
|---|---|---|---|
| **Example** | **Reaction time [h]** | **DOPAC [mM]** | **3-HT [mM]** |
| 5-1 | 0.5 | 7.85 | 0.35 |
| 5-2 | 1 | 8.71 | 0.69 |
| 5-3 | 2 | 5.79 | 2.54 |
| 5-4 | 4 | 3.14 | 6.95 |
| 5-5 | 6 | 1.37 | 10.04 |
| 5-6 | 8 | 0 | 9.78 |
| 5-7 | 16 | 0 | 11.29 |
| 5-8 | 18 | 0 | 9.47 |
| 5-9 | 20 | 0 | 9.73 |
| 5-10 | 22 | 0 | 11.19 |
| 5-11 | 24 | 0 | 10.79 |

**Example 6–influence of cofactors on reaction**

The influence of different cofactors and cofactor concentrations on the reaction of DOPAC to 3-HT was studied under the following conditions: CPE1 and CPE2 resting cell sample aliquots respectively, prepared according to Example 1, were thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with buffer B in case of CPE1, with buffer C in case of CPE2. These cell pellets were dispersed in buffer B in case of CPE1, with buffer C in case of CPE2.

The cofactors listed in Table 6 were added in form of an aqueous solution to give final concentrations as listed in Table 6. Subsequently, 100 mM of DOPAC in water was added to give a final concentration of 10 mM in a total volume of 500 µL. The thus prepared samples in Eppendorf vessels were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm with open lid. After 4 h they were centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm, polypropylene). The filtrates were analyzed with Method A. Table 6 shows the influence of the cofactor composition and concentration, only one example was done with CPE2.

"Relative %" means that the % value of Ex 6-8 was set to 100% and all other % values were normalized to this value.

| Table 6 | | | CPE1 | | CPE2 | |
|---|---|---|---|---|---|---|
| NADPH [mM] | $NADP^+$ [mM] | NADH [mM] | Example | Relative % | Example | Relative % |
| 0 | 0 | 0 | 6-1-CPE1 | 3 | --- | --- |
| 10 | 0 | 0 | 6-2-CPE1 | 37 | --- | --- |
| 20 | 0 | 0 | 6-3-CPE1 | 83 | 6-3-CPE2 | 55 |
| 10 | 0 | 10 | 6-4-CPE1 | 91 | --- | --- |
| 5 | 0 | 5 | 6-5-CPE1 | 22 | --- | --- |
| 0 | 10 | 10 | 6-6-CPE1 | 7 | --- | --- |
| 0 | 0 | 10 | 6-7-CPE1 | 18 | --- | --- |
| 0 | 0 | 20 | 6-8-CPE1 | 100 | --- | --- |
| 0 | 5 | 5 | 6-9-CPE1 | 5 | --- | --- |

**Example 7–cofactor recycling using GDH and glucose**

Recycling of NAD(P)H was studied under the following conditions: CPE1 resting cell sample aliquots, prepared according to Example 1 but with the difference that the protein expression phase after induction was 16 to 18 h instead of 4 h, were used. After this protein expression phase, cells were harvested by centrifugation at 2831 g, 4°C for 10 min. The supernatant was discarded and the cell pellet washed twice with buffer C. These cell pellets were dispersed in buffer C.

GDH and glucose were added to give final concentrations as listed in Table 7. Subsequently, 100 mM DOPAC in water was added to give a final concentration of 10 mM in a total volume of 500 µL. The thus prepared samples in Eppendorf vessels were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm with open lid. After 5 h, the samples were centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm polypropylene). The filtrates were analyzed with Method A. Table 7 shows the influence of cofactor recycling with the system GDH and glucose

1 mg/mL GDH corresponds to 2.7 U for $NADP^+$ reduction (1 U catalyzes the conversion of 1 µmol $NADP^+$ per min)."Relative %" means that the % value of Ex 7-5 was set to 100% and all other % values were normalized to this value.

| Table 7 | | | |
|---|---|---|---|
| Example | GDH [U] | Glucose [mM] | Relative % |
| 7-1 | 0.027 | 10 | 72 |
| 7-2 | 0.135 | 10 | 70 |
| 7-3 | 0.27 | 10 | 97 |
| 7-4 | 0.27 | 20 | 97 |
| 7-5 | 0.27 | 50 | 100 |
| 7-6 | 2.7 | 10 | 83 |
| 7-7 | 13.5 | 10 | 43 |
| 7-8 | 27 | 10 | 61 |
| 7-9 | 27 | 20 | 60 |
| 7-10 | 27 | 50 | 57 |

**Example 8–cofactor recycling using FDH and formate or citrate**

Recycling of NAD(P)H was studied under the following conditions: CPE1 and CPE2 resting cell sample aliquots respectively, prepared according to Example 1, were thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with buffer C. The cell pellets were dispersed in buffer C.

The FDH was used in form of a cell free extract of a *E. coli* Rosetta containing the plasmid pRSF:FDH from Candida boidinii as described in Mädje *et al.*, Microb. Cell Fact., 2012, 11:7, with the described 18 °C during the induction phase which is reported by Mädje to provide FHD in an amount of 28% of the total soluble protein in the extract. FDH and formate were added to give final concentrations as listed in Table 8. Alternatively, citrate was used, with and without extra $NADP^+$ in final concentrations as listed in Table 8.

Subsequently, 100 mM DOPAC in water was added to give a final concentration of 10 mM in a total volume of 500 µL. The thus prepared samples in Eppendorf vessels were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm with open lid. After 5 h, the samples were centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm polypropylene). The filtrates were analyzed with Method A.

Table 8 gives the respective values and results. 1.08 mg/mL CFE containing FDH corresponds to 0.27 Units (1 U catalyzes the conversion of 1 µmol $NAD^+$ per min).

| **Table 8** | | | | **CPE1** | | **CPE2** | |
|---|---|---|---|---|---|---|---|
| **FDH [U]** | **Formate [mM]** | **Citrate [mM]** | **$NADP^+$ [mM]** | **Example** | **%** | **Example** | **%** |
| 0.27 | 10 | --- | --- | 8-1-CPE1 | 65 | 8-1-CPE2 | 36 |
| --- | --- | 10 | --- | 8-2-CPE1 | 26 | --- | --- |
| --- | --- | 10 | 1 | 8-3-CPE1 | 67 | 8-3-CPE2 | 46 |
| --- | --- | 50 | --- | 8-4-CPE1 | 77 | --- | --- |

**Example 9 – strain PE2**

Preparation of pEHISTEV-14-CgPPTase

The DNA of SEQ ID NO: 13 was amplified from genomic DNA of Corynebacterium glutamicum DSM 20300, ATCC 13032 including the introduction of restriction sites NcoI and HindIII using Phusion® High-Fidelity DNA polymerase (Finnzymes) and primers of SEQ ID NO: 23 and of SEQ ID NO: 24. a single colony of Corynebacterium glutamicum was picked with a sterile tooth pick into 50 µL of distilled water. After incubation at 95°C for 10 min and centrifugation for 5 min at room temperature and 13 200 rpm, 5 µL of the supernatant was used as template DNA for the PCR reaction. The PCR reaction was thermally cycled: 98ºC for 30 s, followed by 20 cycles of 98ºC for 10 s, 50ºC for 20 s, and 72ºC for 15 s, then a final incubation of 72ºC for 7 min. The PCR products were gel purified with the QIAquick® Gel Extraction Kit (QIAGEN). The thus obtained PCR product was double digested with NcoI and HindIII restriction enzymes (Fermentas) in the presence of 1x Tango buffer (Fermentas) and column purified according to QIAquick® PCR purification protocol (QIAGEN). The gene was ligated into the pEHISTEV vector (Huanting *et al.* Protein Expression and Purification 2009, 63, 102-111) which was also digested with NcoI and HindIII. The ligation was carried out

overnight at 16°C in the presence of T4 ligase (Fermentas) and T4 ligation buffer (Fermentas). The ligation product was desalted for 40 min on MF-Millipore™ membrane filters (0.025µm filter code VSWP) (Millipore), transformed into electrocompetent *E. coli* TOP10 F' cells and plated out on a LB-plate supplemented with 50 µg Kan/mL. The resulting plasmid, called pEHISTEV-14-CgPPTase, was harvested by GeneJET™ Plasmid Miniprep Kit (Fermentas) and the sequence was confirmed by LGC Genomics.

Transformation with pEHISTEV-14-CgPPTase to provide PE2

60 µL of chemically competent cells *E. coli* BL21 (DE3) Gold were transformed with 100 ng of pEHISTEV-14-CgPPTase.The transformation, selection, using Kan only for selection, and glycerol stock sample preparation were done as described in Example 1 to provide strain PE2 in form of glycerol stock samples.

**Example 10 - 3-HT preparation using CFEs**

CE1, CPE1, PE1, PE2 and CPE2 CFEs of resting cell samples were obtained as follows: For each strain, 20 mL of LB medium containing 100 mg/L Amp or 50 mg/L Kan or both respectively, was inoculated with 5 µL of a respective glycerol stock sample of each strain respectively, prepared according to example 1 and example 9 respectively.

Cultivation was carried out overnight at 37°C and 120 rpm. Subsequently, 500 mL LB medium, containing 100 mg/L Amp or 50 mg/L Kan or both respectively, in sterile 2 L baffled flask were inoculated with pre-culture in such an amount in order to obtain an OD of ca. 0.1. Incubation for 1.5 to 2 h at 37°C and 120 rpm delivered cultures with an OD between 0.4 and 0.6, which were induced with 1 mM IPTG (0.5 mL of 1M aqueous solution). The protein expression phase proceeded for 4 h at 37°C and 120 rpm for strains CE1, PE1, CPE1 and CPE2, and overnight at 25 °C and 120 rpm for strain PE2. Subsequently, cells were harvested by centrifugation at 2831 g at 4 °C for 10 min. The cell pellets were washed twice with Buffer B and centrifuged again at 2831 g at 4 °C for 10 min. The supernatant was removed and the cell pellets were dispersed in Buffer B, sonicated, ultracentrifuged at 107527.5 g and 4 °C for 45 min. The supernatants representing the CFEs were shock frozen in liquid nitrogen and stored at -20 °C.

Thawed CFEs, volumes as given in table 10, were mixed in Eppendorf vessels according to the combinations described in Table 10, 0.5 M NADPH in water, 250 mM ATP in water and 100 mM of DOPAC in water were added to give final concentrations of 20 mM, 10 mM and 10 mM respectively, in a total volume of 500 µL.

The thus prepared samples were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm with closed lid. After overnight incubation, the samples were centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm polypropylene). The filtrates were analyzed with Method A. Table 10 shows the influence of the CFE composition on the reaction.

| Table 10 | | | | | | |
|---|---|---|---|---|---|---|
| **Example** | **CPE1 [µL]** | **CE1 [µL]** | **PE1 [µL]** | **PE2 [µL]** | **CPE2 [µL]** | **%** |
| 10-1 | 200 | --- | --- | --- | --- | 3.7 |
| 10-2 | --- | 200 | --- | --- | --- | 3.1 |
| 10-3 | --- | 200 | 200 | --- | --- | 5.9 |
| 10-4 | --- | 200 | --- | 200 | --- | 16.4 |
| 10-5 | --- | --- | --- | --- | 200 | 6.0 |

**Example 11–reaction condition optimization using citrate and glucose**

In order to optimize the reaction conditions, the 'design of experiments' method (DOE) was used.

CPE1 resting cell sample aliquots respectively, prepared according to Example 1, were thawed and centrifuged. The supernatant was discarded and the cell pellet was washed twice with buffer D. The cell pellets were dispersed in buffer D. Stock solutions of sodium citrate and glucose in buffer D at pH 6.0 were added to give final concentrations as specified in Table 11. The samples were incubated in 1.5 ml reaction tubes for 1h at 28 °C and 1000 rpm with open lids.

After that, DTT, EDTA and magnesium chloride, each dissolved in buffer D at pH 6.0, were added to give final concentrations as specified in Table 11. Subsequently, 300 mM DOPAC in buffer D at pH 6.0 was added to give final concentrations as specified in Table 11 in a total volume of 500 µL. The thus prepared samples in Eppendorf vessels were incubated in an Eppendorf Thermomixer at 28 °C and 1000 rpm with open lid. After 21 h, the samples were centrifuged for 10 min at 28 °C and the supernatants were filtered through a plate filter (0.2 µm polypropylene). The filtrates were analyzed with Method A.

Table 11 gives selected values and results.

| Table 11 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Example** | **DOPAC [mM]** | **citrate [mM]** | **Glucose [mM]** | **DTT [mM]** | **EDTA [mM]** | **$MgCl_2$ [mM]** | **3-HT [mM]** |
| 11-1 | 30 | 55 | 55 | 5.5 | 5.5 | 5.5 | 22.7 |
| 11-2 | 10 | 10 | 10 | 1 | 1 | 1 | 6.8 |
| 11-3 | 10 | 10 | 10 | 10 | 1 | 10 | 6.7 |
| 11-4 | 10 | 10 | 100 | 1 | 10 | 10 | 9.2 |
| 11-5 | 10 | 10 | 100 | 10 | 10 | 1 | 8.6 |
| 11-6 | 10 | 100 | 10 | 1 | 10 | 10 | 7.2 |
| 11-7 | 10 | 100 | 10 | 10 | 10 | 1 | 5.6 |
| 11-8 | 10 | 100 | 100 | 1 | 1 | 1 | 10.4 |
| 11-9 | 10 | 100 | 100 | 10 | 1 | 10 | 10.0 |
| 11-10 | 50 | 10 | 10 | 1 | 10 | 1 | 1.0 |
| 11-11 | 50 | 10 | 10 | 10 | 10 | 10 | 0.8 |
| 11-12 | 50 | 10 | 100 | 1 | 1 | 10 | 2.7 |
| 11-13 | 50 | 10 | 100 | 10 | 1 | 1 | 14.2 |
| 11-14 | 50 | 100 | 10 | 1 | 1 | 10 | 4.6 |
| 11-15 | 50 | 100 | 10 | 10 | 1 | 1 | 3.9 |
| 11-16 | 50 | 100 | 100 | 1 | 10 | 1 | 3.4 |
| 11-17 | 50 | 100 | 100 | 10 | 10 | 10 | 2.0 |
| 11-18 | 30 | 100 | 10 | 5 | 4.2 | 1.1 | 11.2 |
| 11-19 | 30 | 67 | 58 | 1 | 5.5 | 4.9 | 28.3 |

**Example 12: Reaction on Preparative Scale**

120 mL of LB medium containing 100 mg/L Amp and 50 mg/L Kan, respectively, was inoculated with a single colony of CPE1 grown on LB-plates containing 100 mg/L Amp and 50 mg/L Kan, respectively; this single colony was prepared according to example 1 described after the sentence "The strains were selected as follows:" and before the sentence "Preparation of glycerol stock samples:".

Cultivation was carried out overnight at 37°C and 120 rpm. Subsequently, 6 times 430 mL LB medium, containing 100 mg/L Amp and 50 mg/L Kan respectively, in sterile 2 L baffled flask were inoculated with pre-culture in such an amount in order to obtain an OD of ca. 0.1. Incubation for 1.5 to 2 h at 37°C and 110 rpm delivered cultures with an OD between 0.4 and 0.6, which were induced with 1 mM IPTG (0.45mL of 1 M aqueous solution). The protein expression phase proceeded for 4 h at 37°C and 110 rpm. Subsequently, cells were harvested by centrifugation at 2831 g, 4°C for 10 min. The cell pellet was washed twice with buffer C, and centrifuged again at 2831 g, 4°C for 10 min. The supernatant was removed and the cell pellet was dispersed in 50% glycerol and shock-frozen as 40 mL aliquots in liquid nitrogen. These resting cell samples were stored at -80°C. One aliquot of CPE1 resting cells was thawed and centrifuged. The supernatant was discarded and the cell pellet washed twice with buffer C. a stirring bar was added and the cell pellet (3 g wet weight) was dispersed in buffer D in a 500 mL polypropylene (PPCO) beaker. Stock solutions of sodium citrate and glucose in buffer D at pH 6.0 were added to give final concentrations (based on the total reaction volume or 100 mL) of 10 mM and 100 mM, respectively. The sample was incubated for 1h at 28 °C and 100 rpm in the open PPCO beaker on a magnetic stirrer.

After that, DTT, EDTA and magnesium chloride, each dissolved in buffer D at pH 6.0, were added to give final concentrations of 1 mM, 10 mM and 10 mM, respectively. Subsequently, 300 mM DOPAC in buffer D at pH 6.0 was added to give a final concentration of 10 mM in a total volume of 100 mL. The thus prepared suspension in the PPCO beaker (called "first PPCO beaker") was covered with gauze and incubated on a magnetic stirrer at 28 °C and 100 rpm stirring speed. 200 µL samples were withdrawn periodically (see column "Time" in Table 12), centrifuged for 10 min at room temperature and the supernatants were filtered through a plate filter (30 kDa cut off, modified polyethersulfone). The filtrates were analyzed with Method A.

After 23 h and 45 h, new portions of resting cells (3.0 g and 2.9 g wet cell weight) were prepared as follows: One aliquot of CPE1 resting cells, prepared according to this example, was thawed on ice, diluted with buffer C and centrifuged. The supernatant was discarded and the cell pellet was washed two more times with buffer C. The cell pellet was dispersed in buffer D in a PPCO beaker. Stock solutions of sodium citrate (1.5 mL, 0.67 M) and glucose (10 mL, 1 M), both in buffer D at pH 6.0, were added to the cell pellet. The sample was incubated for 1h at 28 °C and 90 rpm in the open vessel in a rotary shaker. The mixture was added to the first PPCO beaker with the ongoing DOPAC reduction reaction and the stirring speed was increased to 200 rpm after the first addition of fresh cells. The second addition was carried out likewise.

After 144 h, the reaction was stopped by centrifugation at 15344 g. The pellet was first washed with 25 ml of buffer D by disperging and centrifugation, and then additionally two times with 10 ml

of buffer D. The pH of the combined supernatants was adjusted to pH 2.0 with concentrated HCl and the compounds were extracted with ethyl acetate four times. The combined extracts (175 mL) were dried with sodium sulfate and the solvent was removed at 135 mbar at 40°C. The residue was purified by silica gel chromatography using 15 g of silica gel and dichloromethane: MeOH of 15:1 with 0.15% of triethylamine as the eluent. The product containing fractions were pooled and the solvent was removed under reduced pressure to give 29.2 mg of a yellowish oil. Product purity was confirmed with Method a to be > 99% and product identity was confirmed by comparison of chromatographic behavior and by $^{1}H$ and $^{13}C$ NMR against standard 3-HT.

| **Table 12** | | | |
|---|---|---|---|
| **Entry** | **Time [h]** | **Bioconversion [%]** | **3-HT [mM]** |
| 12-1 | 3 | 0.9 | 0.1 |
| 12-2 | 20 | 11.3 | 1.2 |
| 12-3 | 23 | 13.0 | 1.3 |
| 12-4 | 45 | 19.7 | 1.8 |
| 12-5 | 92 | 32.5 | 2.7 |
| 12-6 | 95 | 33.6 | 3.0 |
| 12-7 | 116 | 42.7 | 4.4 |
| 12-8 | 144 | 38.7 | 3.8 |

**Sequence Listing Free Text**

<210> 9

<223> *Nocardia* sp. NRRL 5646 ATP/NADPH-dependent carboxylic acid reductase (car), EMBL Nucleotide Sequence Database accession no. AY495697, codon optimized for E.coli

<210> 15

<223> Gene sequence of plasmid pJexpress-404-9-CAR

<210> 16

<223> Gene sequence of plasmid pJexpress-401-11-EcPPTase

<210> 17

<223> Primer 1

<210> 18

<223> Primer 2

<210> 19

<223> Primer 3

<210> 20

<223> Primer 4

<210> 21

<223> Primer 5

<210> 22

<223> Primer 6

<210> 23

<223> Primer 7

<210> 24

<223> Primer 8

### 7.4.4 Claims

1. Method for the preparation of 3-hydroxytyrosol by a reaction, the reaction comprises an enzymatic reduction of 3,4-dihydroxyphenyl acetic acid,

wherein the enzymatic reduction is done using two enzymes, a carboxylic acid reductase CAR and a 4'-phosphopantetheinyl transferase PPTase;

wherein

CAR comprises each of the three amino acid sequences SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5 with 70 to 100% identity;

PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14, each sequence is comprised with 70 to 100% identity.

2. Method according to claim 1, wherein CAR comprises each of the three amino acid sequences SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

each sequence is comprised with 70 to 100% identity.

3. Method according to claim 1, wherein CAR comprises each of the three amino acid sequences SEQ ID NO: 2, SEQ ID NO: 10 and SEQ ID NO: 6;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

each sequence is comprised with 70 to 100% identity.

4. Method according to claim 1, wherein CAR comprises the CAR from *Nocardia* sp. strain NRRL 5646;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14;

the CAR from *Nocardia* sp. strain NRRL 5646 and each of the sequences SEQ ID NO: 12 and SEQ ID NO: 14 is comprised with 70 to 100% identity.

5. Method according to claim 1, wherein CAR comprises the sequence SEQ ID NO: 7;

and PPTase comprises the sequence SEQ ID NO: 12 or comprises the sequence SEQ ID NO: 14,

each sequence is comprised with 70 to 100% identity.

6. Method according to claim 1, wherein CAR has the sequence SEQ ID NO: 7;

and PPTase has the sequence SEQ ID NO: 12 or has the sequence SEQ ID NO: 14;

each sequence with 70 to 100% identity.

7. Method according to claim 1, wherein CAR has the sequence SEQ ID NO: 7;

and PPTase has the sequence SEQ ID NO: 12 or has the sequence SEQ ID NO: 14;

each sequence with 80 to 100% identity.

8. Method according to claim 1, wherein CAR has the sequence SEQ ID NO: 7;

and PPTase has the sequence SEQ ID NO: 12 or has the sequence SEQ ID NO: 14;

each sequence with 90 to 100% identity.

9. Method according to claim 1, wherein CAR has the sequence SEQ ID NO: 7;

and PPTase has the sequence SEQ ID NO: 12 or has the sequence SEQ ID NO: 14;

each sequence with 95 to 100% identity.

10. Method according to one or more of claims 1 to 9, wherein the sequence SEQ ID NO: 12 is the embodiment for PPTase.

11. Method according to one or more of claims 1 to 10, wherein CAR and PPTase are used in the reaction

- in form of a cell free extract of a microorganism containing one of the enzymes or both;
- in form of a microorganism, that expresses or expressed both enzymes, or in form of two mircoorganisms, one microorganism expressing the one enzyme and the other microorganism expressing the other enzyme;
- in form of parts of a microorganism, that expressed one of the enzymes or both; or
- as a combination of these embodiments.

12. Method according to one or more of claims 1 to 11, wherein CAR and PPTase are used in the reaction in form of a microorganism, that expresses both enzymes, or in form of two mircoorganisms, one microorganism expressing the one enzyme and the other microorganism expressing the other enzyme, or in form of a cell free extract containing the enzymes.

13. Method according to claim 11 or 12, wherein the microorganism is selected from the group of families consisting of *Enterobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Streptomycetaceae* and *Nocardiaceae*.

14. Method according to one or more of claims 11 to 13, wherein the mircoroganism is selected from the group consisting of *Escherichia, Bacillus, Lactobacillus, Pseudomonas, Streptomyces, Nocardia, Burkholderia, Salmonella, Agrobacterium* and *Rhodococcus.*

15. Method according to one or more of claims 11 to 13, wherein the microorganism is *Escherichia coli.*

16.Method according to one or more of claims 1 to 15, wherein the reaction is done in the presence of a buffer (REAC);

buffer (REAC) is selected from the group consisting of phosphate buffer, a buffer based on 4-2-hydroxyethyl-1-piperazineethanesulfonic acid, a buffer based on 2-{[tris(hydroxymethyl)methyl]-amino}ethanesulfonic acid, a buffer based on 3-(N-morpholino)propanesulfonic acid, a buffer based on piperazine-N,N'-bis(2-ethanesulfonic acid, a buffer based on 2-(N-morpholino)ethanesulfonic acid and mixtures thereof.

17. Method according to claim 16, wherein buffer (REAC) is phosphate buffer, 2-(N-morpholino)ethanesulfonic acid or mixtures thereof.

18. Method according to claim 16 or 17, wherein the reaction is started in the presence of buffer (REAC) with a pH of from 5 to 8.5.

19. Method according to one or more of claims 1 to 18, wherein the reaction is done in the presence of DTT.

20. Method according to one or more of claims 1 to 19, wherein the reaction is done in the presence of EDTA.

21. Method according to one or more of claims 1 to 20, wherein the reaction is done in the presence of a cofactor, the cofactor is selected from the group consisting of NADPH and NADH.

22. Method according to one or more of claims 1 to 21, wherein the reaction is

(i) done in the presence of an enzyme ENZREGENCOF, that regenerates the cofactor, and a respective substrate REGENSUB, that is used by ENZREGENCOF to regenerate the cofactor; or

(ii) the reaction is done using electrical current via redox electrodes for the regeneration of the cofactor; or

(iii) the reaction is done in the presence of a redox mediator for the regeneration of the cofactor; or

(iv) done in the presence of a substrate (SUBSCIT), substrate (SUBSCIT) is a substrate of the citric acid cycle or glucose.

23. Method according to claim 22, wherein the reaction is

(i) done in the presence of an enzyme ENZREGENCOF, that regenerates the cofactor, and a respective substrate REGENSUB, that is used by ENZREGENCOF to regenerate the cofactor; or

(iv) done in the presence of a substrate (SUBSCIT).

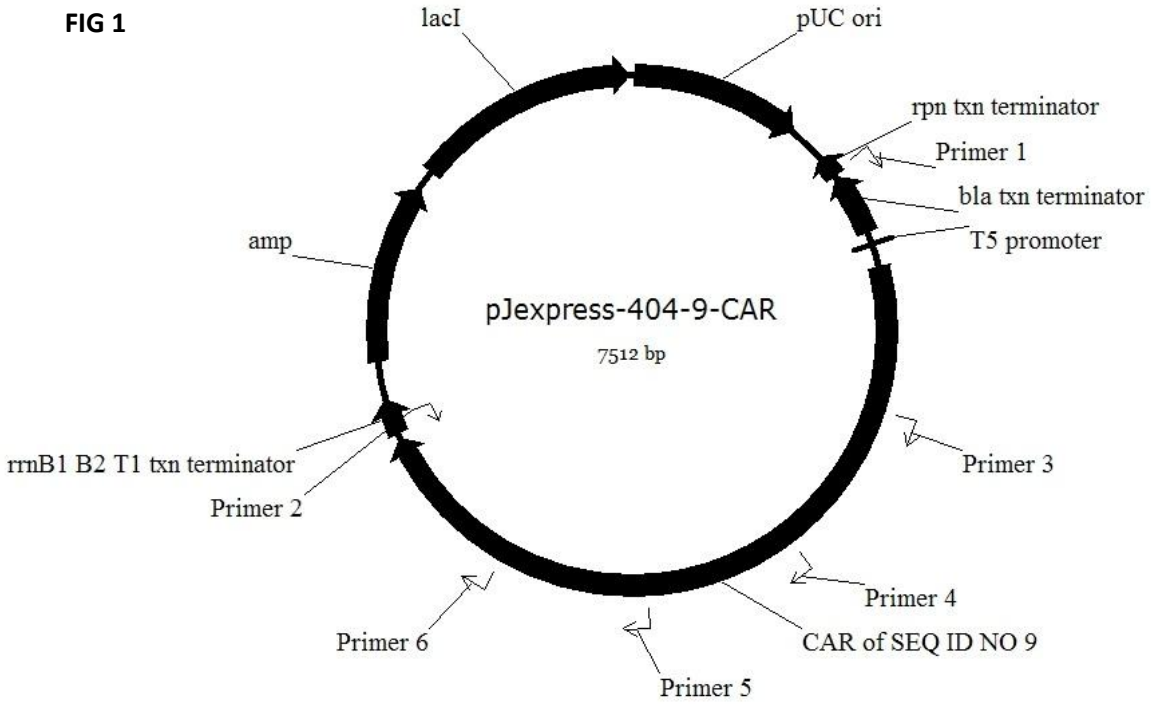
24. Method according to claim 22 or 23, wherein ENZREGENCOF is selected from the group consisting of glucose 1-dehydrogenase, formate dehydrogenase, phosphonate dehydrogenase, NAD(P) oxidase, alcohol dehydrogenase and mixture thereof;

and REGENSUB is glucose, formate, phosphonate, hydrogen peroxide or an alcohol as substrate for the respective ENZREGENCOF.

25. Method according to one or more of claims 22 to 24, wherein ENZREGENCOF is GDH with REGENSUB being glucose or ENZREGENCOF is FDH with REGENSUB being formate or ENZREGENCOF is a mixture of GDH and FDH with REGENSUB being glucose and formate.

26. Method according to one or more of claims 1 to 25, wherein the reaction is done in the presence of a kation KAT, KAT is selected from the group consitsing of $Mg^{2+}$, $Zn^{2+}$ and mixtures thereof.

**FIG 1**


lacI
pUC ori
rpn txn terminator
Primer 1
bla txn terminator
T5 promoter
amp
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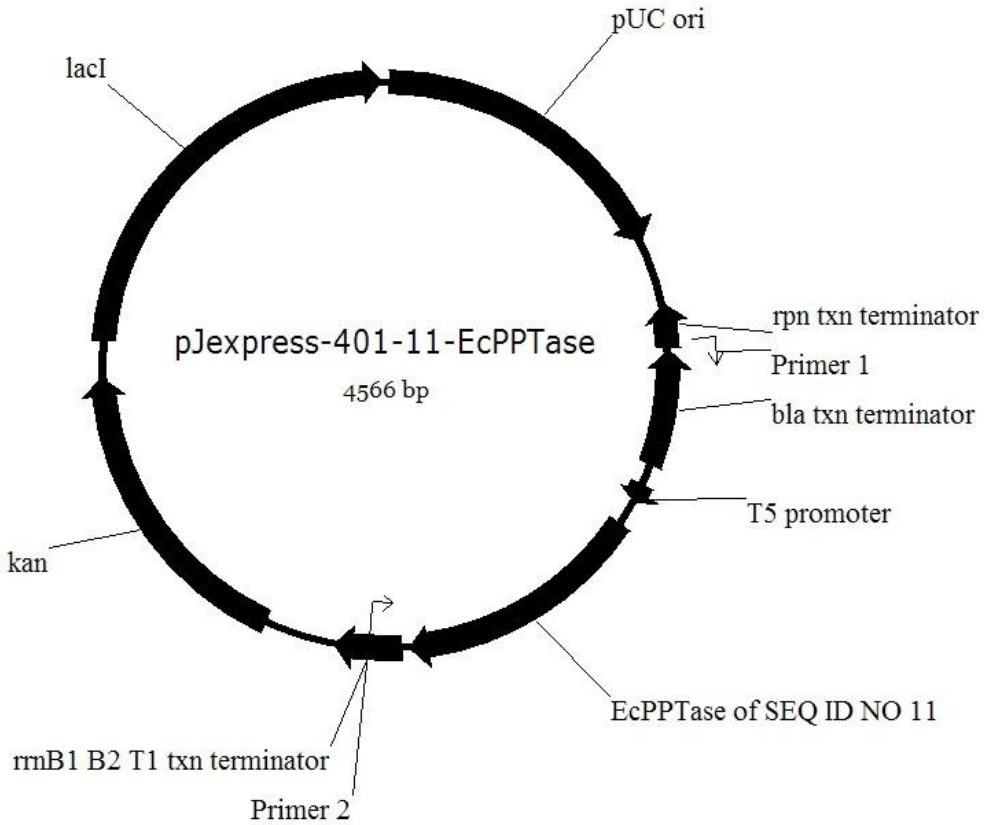
Primer 4
Primer 6
CAR of SEQ ID NO 9
Primer 5

**FIG 2**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T5 promoter
kan
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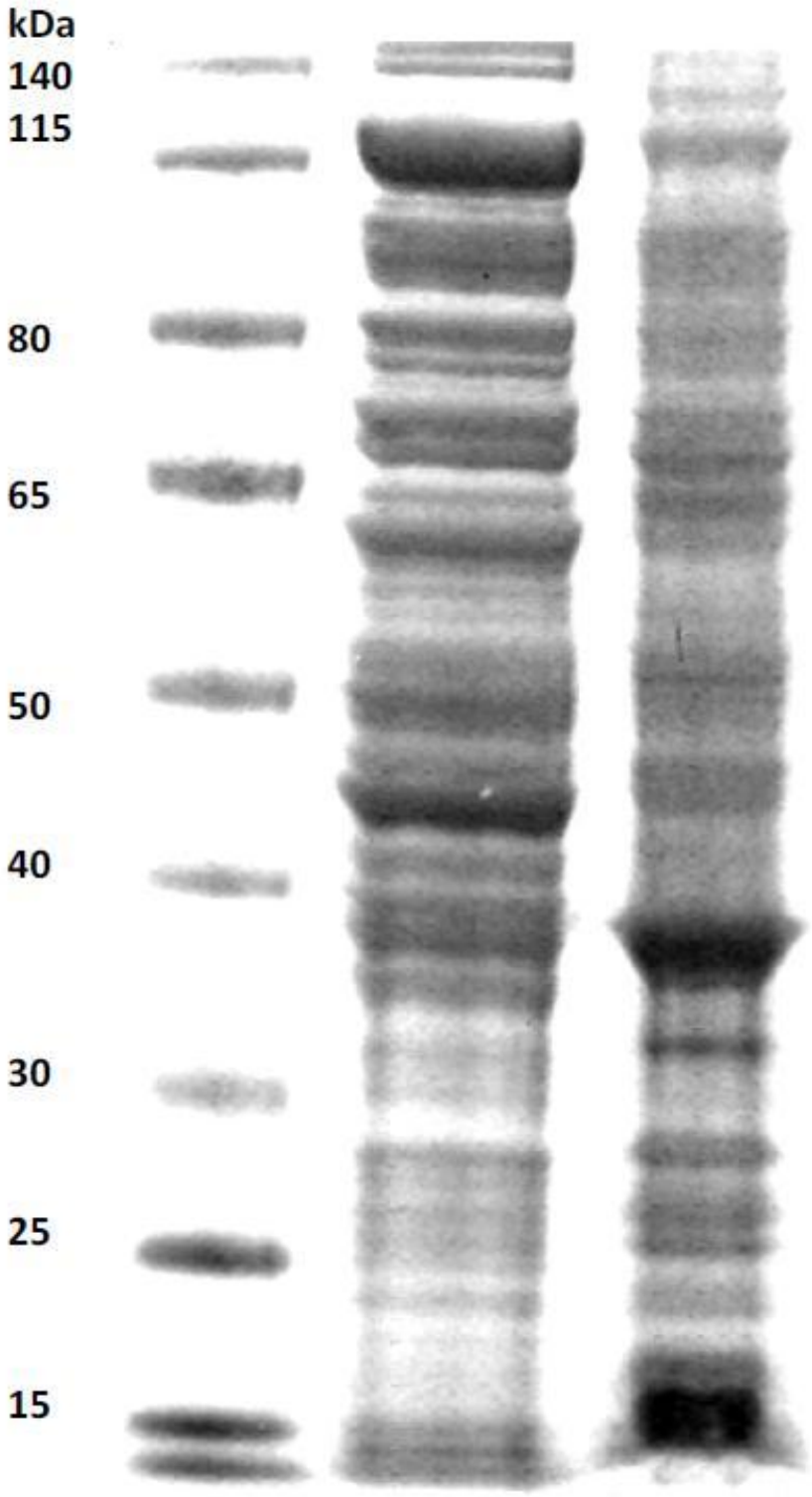
rrnB1 B2 T1 txn terminator
Primer 2

**FIG 3**



# 8 Appendix

## 8.1 European Curriculum Vitae

**PERSONAL INFORMATION**

| | |
|---|---|
| Name | **NAPORA-WIJATA, KAMILA** |
| Address | **Koßgasse 16/90, 8010 Graz, Austria** |
| Telephone | **(+43) 681 1066 1019** |
| E-mail | **kama_n@wp.pl** |
| Nationality | Polish |
| Date of birth | 04.03.1985 |

**WORK EXPERIENCE**

September 2010 - December 2013

**Junior researcher** at the Austrian Centre of Industrial Biotechnology, Graz, Austria, www.acib.at

Tasks/Functions:

- *DNA analysis (DNA isolation, gel electrophoresis, cloning)*
- *Heterologous protein expression*
- *Protein analysis and characterization (SDS-PAGE, BCA and Bradford protein assay, enzymatic assays)*
- *Bioconversion with whole cells as well as purified enzymes*
- *TLC and HPLC analysis*
- *Work documentation and presentation of results (reports, posters and oral presentations)*
- *Laboratory supervision of trainees and bachelor students*

February 2010

**Trainee** at the Finepharm, Jelenia Góra, Poland, www.finepharm.pl

Tasks/Functions:

- *Analysis of initial materials*
- *Determination of enzyme activity using the visosimeter*
- *Become acquainted with capillary electrophoresis and TLC*

July 2009 – September 2009

February 2009

**Trainee** at the Christian Doppler Laboratory for Allergy Research, Vienna, Austria, www.meduniwien.ac.at/allergy-research-christian-doppler/index.php

Tasks/Functions:

- *Expression and purification of viral recombinant proteins and their analysis by SDS-PAGE, Western Blotting, BCA protein assay and ELISA*
- *Removal of endotoxins from received proteins*
- *Synthesis of viral peptides, their purification by HPLC and verification with MALDI*

**EDUCATION AND TRAINING**

| | |
|---|---|
| October 2010 – present | **PhD student** in the field of Biotechnology at the Graz University of Technology, Austria<br>Dissertation title: *Identification and expression of new microbial oxidoreductases* |
| October 2005 – July 2010 | **Master of Science** in Biotechnology with specialization in Fermentation Technology and Technical Microbiology at the Lodz University of Technology, Poland<br>Master thesis title: *Secretory proteins of Bacillus cereus group* |
| October 2008 – June 2009 | **Erasmus student** at the University of Applied Sciences, Vienna, Austria |
| September 2000 – June 2005 | Technical School of the Environmental Protection, Radomsko, Poland<br>Specialization: Waste management |

**PERSONAL SKILLS AND COMPETENCES**

MOTHER TONGUE **POLISH**

OTHER LANGUAGES

**ENGLISH**

- Reading skills: very good
- Writing skills: good
- Verbal skills: good

**GERMAN**

- Reading skills: very good
- Writing skills: good
- Verbal skills: good

COMMUNICATIONS AND ORGANISATIONAL SKILLS AND COMPETENCES

- Good communication skills gained during co-organisation of the 10th DocDay
- Good organization skills gained during working as junior researcher in ACIB (conducting several experiments parallel) and during co-organisation of the 10th DocDay
- Leading skills gained during supervision of Erasmus trainees and bachelor students and supported by completion of the course: "Leading of technical teams"

TECHNICAL SKILLS AND COMPETENCES

- Good command of Microsoft Office™ tools and Reference manager: Mendeley
- Good command of Adobe Photoshop CS4 tools gained during the courses: "Work with Adobe InDesign" and "Work with Adobe Illustrator" and applied during design of graphical abstracts
- Good command of Vector NTI, Laser DNA 8.0, CLC Main Workbench, HPLC ChemStation software

DRIVING LICENCE(S) B

## 8.2 List of publications

### 8.2.1 Papers

1. Kamila Napora-Wijata, Gernot A. Strohmeier, Margit Winkler. (2014). Biocatalytic reduction of carboxylic acids. *Biotechnology Journal*, submitted.
2. Kamila Napora-Wijata, Karen Robins, Antonio Osorio-Lozada, Margit Winkler. (2013). Whole cell carboxylate reduction for the synthesis of 3-hydroxytyrosol. *ChemCatChem*, DOI: 10.1002/cctc.201300913
3. Kamila Napora-Wijata, Gernot A. Strohmeier, Manoj N. Sonavane, Manuela Avi, Karen Robins, Margit Winkler. (2013). Enantiocomplementary *Yarrowia lipolytica* oxidoreductases: Alcohol dehydrogenase 2 and short chain dehydrogenase/reductase. *Biomolecules*, 3, 449-460.
4. Kamila Napora, Tanja M. Wrodnigg, Patrik Kosmus, Martin Thonhofer, Karen Robins and Margit Winkler. (2013). *Yarrowia lipolytica* dehydrogenase/reductase: An enzyme tolerant for lipophilic compounds and carbohydrate substrates*. Bioorganic & Medicinal Chemistry Letters,* 23, 3393-3395.

### 8.2.2 Patent application

Patent application pending, PCT/EP2013/076327

Margit Winkler, Kamila Napora-Wijata, Antonio Osorio-Lozada, Karen Robins, Christiaan Rijksen. Method for preparation of 3-hydroxytyrosol.

### 8.2.3 Oral presentations

1. Kamila Napora-Wijata, Manoj N. Sonavane, Jacek Plewka, Dorota K. Pomorska, Karen Robins, Antonio Osorio-Lozada, Kerstin Steiner, Helmut Schwab, Margit Winkler. Carboxylate reductase – a novel route for 3-hydroxytyrosol production, ACIB Science Days 11.09.2013, Graz, Austria.
2. Kamila Napora-Wijata, Manoj N. Sonavane, Jacek Plewka, Dorota K. Pomorska, Karen Robins, Antonio Osorio-Lozada, Kerstin Steiner, Helmut Schwab, Margit Winkler. Carboxylate reductase – a novel route for 3-hydroxytyrosol production, 15. Chemietage, 25.09.2013, Graz, Austria.
3. Kamila Napora, Tanja Wrodnigg, Patrick Kosmus, Martin Thonhofer, Manoj N. Sonavane, Dorota K. Pomorska, Thomas Prossliner, Karen Robins, Margit Winkler: Mannitol dehydrogenase as a tool for enzymatic oxidation of lipophilic substrates: alcohols and sugar alcohols. 9$^{th}$ DocDay TU Graz, 08.02.2013, Graz, Austria.

### 8.2.4 Posters

1. Kamila Napora-Wijata, Manoj N. Sonavane, Jacek Plewka, Dorota K. Pomorska, Karen Robins, Antonio Osorio-Lozada, Kerstin Steiner, Helmut Schwab, Margit Winkler. Carboxylate reductase – a novel route for 3-hydroxytyrosol production. Biotrans 2013 21-25.07.2013, Manchester, England.

2. Kamila Napora-Wijata, Manoj N. Sonavane, Jacek Plewka, Dorota K. Pomorska, Karen Robins, Antonio Osorio-Lozada, Kerstin Steiner, Helmut Schwab, Margit Winkler: Carboxylate reductase – a novel route for 3-hydroxytyrosol production. 10$^{th}$ DocDay TU Graz, 12.07.2013, Graz, Austria.

3. Kamila Napora, Tanja Wrodnigg, Patrick Kosmus, Martin Thonhofer, Manoj N. Sonavane, Dorota K. Pomorska, Thomas Prossliner, Karen Robins, Margit Winkler. Mannitol dehydrogenase as a tool for enzymatic oxidation of lipophilic substrates: alcohols and sugar alcohols. RPP7- Seventh conference on recombinant protein production, 6-8.03.2013, Laupheim, Germany.

4. Kamila Napora, Natalia Pankiewicz, Manoj N. Sonavane, Stefan Krahulec, Patricia Wildberger, Andrea Camattari, Harald Pichler, Manuela Avi, Margit Winkler. Recombinant expression and characterization of a zinc-dependent alcohol dehydrogenase from *Yarrowia lipolytica*. ACIB Science Days, 2-4.07.2012, Vienna, Austria.

5. Kamila Napora, Natalia Pankiewicz, Manoj N. Sonavane, Stefan Krahulec, Patricia Wildberger, Andrea Camattari, Harald Pichler, Manuela Avi, Margit Winkler. Recombinant expression and characterization of a zinc-dependent alcohol dehydrogenase from *Yarrowia lipolytica*. 8$^{th}$ DocDay TUGraz, 13.07.2012, Graz, Austria.

6. Kamila Napora, Natalia Pankiewicz, Manoj N. Sonavane, Stefan Krahulec, Patricia Wildberger, Andrea Camattari, Harald Pichler, Manuela Avi, Margit Winkler Recombinant expression and characterization of a zinc-dependent alcohol dehydrogenase from *Yarrowia lipolytica*. 6th International Congress on Biocatalysis - Biocat 2012, 2.9.2012 - 6.9.2012, Hamburg, Germany.

7. Kamila Napora, Andrea Camattari, Harald Pichler, Stefan Krahulec, Peter Macheroux, Margit Winkler. Identification of new microbial oxidoreductases. ACIB Science Days, 6-7.04.2011, Alpbach, Austria.

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3. Historical Introduction: Report from the introduction to Enzyme Nomenclature. Retrieved October 30, 2013, from http://www.chem.qmul.ac.uk/iubmb/enzyme/history.html

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10. Napora-Wijata, K., Strohmeier, G., Sonavane, M., Avi, M., Robins, K., & Winkler, M. (**2013**). Enantiocomplementary *Yarrowia lipolytica* oxidoreductases: Alcohol dehydrogenase 2 and short chain dehydrogenase/reductase. *Biomolecules*, *3*(3), 449–460.

11. Napora, K., Wrodnigg, T. M., Kosmus, P., Thonhofer, M., Robins, K., & Winkler, M. (**2013**). Yarrowia lipolytica dehydrogenase/reductase: An enzyme tolerant for lipophilic compounds and carbohydrate substrates. *Bioorganic & Medicinal Chemistry Letters*, *23*, 3393–3395.

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# Table of Figures[7]



[7] This table does not contain figures, which are part of submitted or published papers and patent application.

# List of Tables[8]



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