# Establishment of an Amination Cascade employing Flavin dependent Oxidase and ω-Transaminase

Diplomarbeit
vorgelegt
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Zur Erlangung des akademischen Grades eines Master of Science an der Naturwissenschaftlichen Fakultät der Universität Graz

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"Why not? Why not? Why not not, then, if the best reasoning you can contrive is why not?"

' David Foster Wallace, *Infinite Jest*

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## Abstract

To establish a biotransformation of an alcohol to the corresponding amine, a multienzyme reaction in a cascade fashion was created. The system contained five enzymes using a flavin dependent oxidase as an oxidising agent and a ω-transaminase (ω-TA) as the reductive aminating enzyme in combination with the required recycle enzymes.

After successful overexpression in *Escherichia coli* of several oxidases and ω-transaminases and first activity tests, the most promising candidates turned out to be a long chain alcohol oxidase (LCAO) from *Aspergillus fumigatus* in combination with a ω-transaminase from *Vibrio fluvialis.* Those enzymes were chosen to establish a cascade system using 1-hexanol as test substrate to furnish the corresponding primary amine. An alanine dehydrogenase regeneration system for the amine donor pushed by a glucose dehydrogenase/glucose system and an 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)/horse radish peroxidase (ABTS/HRP) system for the removal of hydrogen peroxide were introduced.

The system was optimised with the introduction of the ω-transaminase from *Chromobacterium violaceum*, pH studies were performed, the oxygen supply and the ratio of the oxidising and the aminating enzymes was optimised and the substrate loading was tested in order to improve the overall outcome of the cascade system. Furthermore, the catalase from *Micrococcus lysodeiktikus* was introduced instead of the HRP/ABTS assay. Finally, time studies were performed monitoring the reaction over time.

Since the substrate scope represents an important feature of a biocatalytic process, the substrate tolerance of the LCAO/cascade system was investigated applying a broad range of structurally different alcohols. Aliphatic alcohols and ω-halogenated aliphatic alcohols were transformed with good to excellent conversions to the corresponding amines, whereas unsaturated, benzylic or secondary alcohols were not accepted by the enzymatic system. To prove that the biotransformation is performed by the cascade enzymes, control studies with “empty” *E. coli* host cells were performed. Furthermore, the over-oxidation to the corresponding carboxylic acid, which occurred as minor side reaction was investigated.

Finally, a sequence alignment were performed for LCAO from *A. fumigatus* with a sequence identity of 70% for LCAO from *Aspergillus terreus*.

# Kurzfassung

Um eine Biotransformation eines Alkohols in ein Amin zu bewerkstelligen wurde eine Multienzym-Reaktionskaskade konzipiert. Dieses System beinhaltet fünf verschiedene Enzyme und verwendet als oxidierende Spezies eine flavinabhängige Oxidase und für die reduktive Aminierung eine ω-Transaminase in Kombination mit den benötigten Recycling-Enzymen.
Nach der erfolgreichen Überexpression einer Reihe von Oxidasen und ω-Transaminasen in *Escherichia coli*, waren die vielversprechensten Kandidaten eine long chain alcohol oxidase (LCAO) aus *Aspergillus fumigatus* in Kombination mit der ω-Transaminase aus *Vibrio fluvialis*. Diese Enzyme wurden ausgewählt um ein Kaskadensystem zu etablieren mit 1-Hexanol als Testsubstrat um das entsprechende Amin zu erhalten. Ein Alanin Dehydrogenase Regenerationsystem für den Amindonor, angetrieben durch ein Glukose Dehydrogenase/Glukose System und 2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure) /Merrettichperoxidase (ABTS/HRP) um Wasserstoffperoxid zu spalten, wurden in das System eingebracht.
Um das Kaskadensystem zu optimieren wurden folgenden Studien durchgeführt: (i) Einführung der ω-Transaminase (ω-TA) aus *Chromobacterium violaceum*, (ii) pH Studien, (iii) Verhältnis des oxidierenden und aminierenden Enzyms, (iv) Beladung mit Substrat um eine mögliche Inhibierung zu vermeiden (v) die Verwendung einer Katalase aus *Micrococcus lysodeiktikus* anstatt des ABTS/HRP Assays und (vi) Zeitstudien um die Reaktion über die Zeit zu verfolgen.
Da das Substratspektrum eine wichtige Eigenschaft eines biokatalytischen Prozesses darstellt, wurde die Substrattoleranz des LCAO/Kaskadensystem unter Verwendung eines breiten Spektrums strukturell unterschiedlicher Alkohole untersucht. Aliphatische Alkohole und ω-halogenierte aliphatische Alkohole wurden mit guten bis exzellenten Werten in die entsprechenden Amine umgewandelt, wohingegen ungesättigte, benzylische und sekundäre Alkole vom Enzymsystem nicht akzeptiert wurden. Um zu überprüfen, ob die Biotransformation auch tatsächlich von den Kaskadenenzymen durchgeführt wurde, wurden Kontrollexperimente mit „leeren“ *E. coli* Zellen durchgeführt. Des Weiteren wurde die Überoxidation zu der entsprechenden Carbonsäure, welche als geringfügige Nebenreaktion auftrat,untersucht.
Abschließend wurde ein Sequenzalignment für LCAO aus *A. fumigatus* durchgeführt, die eine Sequenzidentität von 70% für LCAO aus *Aspergillus terreus* zeigte.

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# 1. Introduction

The use of enzymes as catalysts in chemical reaction is the preferred way that Nature chose to synthesise complex biomolecules.[1] The huge variety of substance classes and their characteristics (polarity, functionalisation, etc.) lead to a variety of different enzymes. With the discovery that these enzymes not only transform their natural substrates, but show a so called „substrate promiscuity“ the foundation for the field of biocatalysis was layed.[2] [3] Catalysis is of high demand in chemical synthesis, where 400 billion Euro product value is generated.[1]

The exploitation of enzymes for the use in synthesis has had to overcome many prejudices. Here, especially the low robustness, the narrow substrate spectra and high cost of the enzymes were criticised. Further applications of biocatalysis require knowledge in the field of biosciences and preparative organic synthesis.

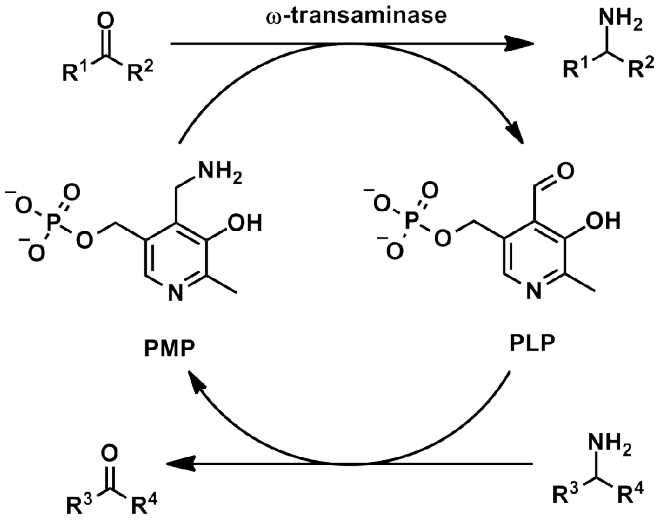
Lipases were one of the first class of enzymes which were investigated in the field of biocatalysis. It proved a lot of prejudice wrong as it showed nearly all features that you could hope from a synthetic catalyst. This lead to numerous applications of the lipase processes in industry.[4]

Enzymes are highly selective in terms of regio-, chemo- and enantioselectivly. Therefore in synthesis, high demanding multistep reactions could be avoided.[2] An integration of biocatalytic steps in total synthesis to chemoenzymatic processes is of high interest, as the latter would lead to cost effective and rather more important sustainable and eco-friendly synthesis in the so called “white” biotechnological and chemical industry.[5]

## 1.1. Amination of ketones by ω–transaminases

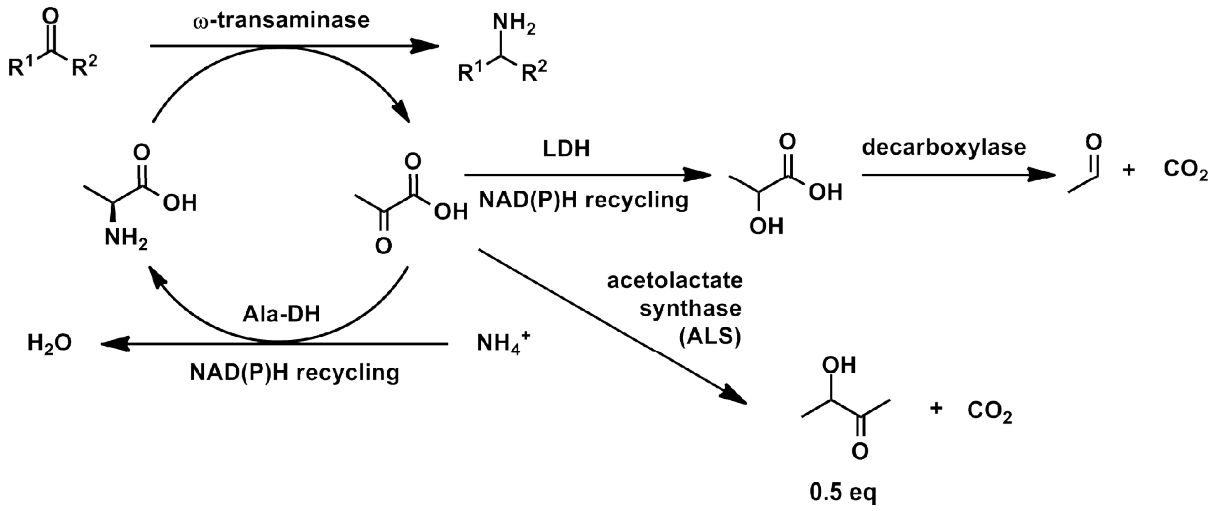
One group of transferases are the ω-transaminases (ω-TA). These enzymes have an advantage over the α-transaminases as they do not need an α-amino acid and a α-keto acid as amino donor and substrate, respectively.[6] Transaminases require as cofactor pyridoxal-5-phosphate (PLP). The pyridine derivative is the activated form of vitamin $B_6$ and is ubiquitous in nature and is present in microorganisms and higher organisms where it plays a role in several important reactions, most prominent transamination and decarboxylation.[7]

The mechanism consists of two half reactions and proceeds like a classic ping-pong bi-bi pattern. The cofactor switches between an aldehyde and the amino moiety pyridoxamine-5'-phosphate (PLP and PMP). The first half reaction creates the PMP-enzyme complex and the amine donor leaves the active site of the enzyme. The second half reaction forms the amine from the corresponding amine acceptor and restores the PLP (Scheme 1).[8] ]9]




**Scheme 1: Reaction of ω-transaminases showing the two half reactions[8] [9]**

ω-TAs were identified around 50 years ago.[10] Although ω-TAs are used for reductive amination, a theoretical conversion of 100% is possible even if the equilibrium is not favourable. So it has to be shifted to product formation. This has been a main goal for scientific research in the last years. Different amine donors were introduced. They have to be employed in high excess or the ketone moiety formed in the side reaction has to be removed from the reaction mixture. Several methods were established [recycling alanine with alanine dehydrogenase, reducing the ketone, further decarboxylation as well as condensation of pyruvate (Scheme 2)].[6] The use of ion exchanger and cyclisation of the amine-product are imaginable.[11]



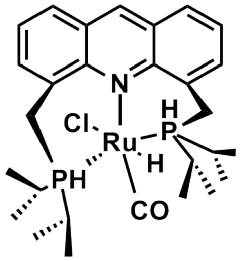

**Scheme 2: Removing and recycling of the amine donor**

If the amine donor is used in high excess, no additional enzyme is needed, but inhibition of the active site due to the amine or the formed ketone might occur. Furthermore, even a high excess is not a guarantee for complete conversion. For some donors, such as 2-propylamine, the co-product acetone can be easily removed due to its volatility. This is not the case for pyruvate when L-alanine is used as donor. One method is the removal of pyruvate from the system with lactate dehydrogenase (LDH). It reduces the keto acid to the corresponding hydroxy acid. The main disadvantage is the further need of the cofactor NADH. Whilst NADH is able to be recycled with enzymes such as formate dehydrogenase (FDH) or glucose dehydrogenase (GDH), it leads to a three-enzyme system. Another possibility, which works without a redox cofactor, is the use of a decarboxylase which forms acetaldehyde and $CO_2$. The donor is completely removed from the reaction mixture, however, the formed acetaldehyde can also be also a substrate for the ω–transaminase and form the corresponding amine. In addition, acetolactate synthase (ALS) can condense two pyruvate molecules to acetoin after decarboxylation. This also leads to an irreversibly shifted equilibrium. Finally, the use of alanine dehydrogenase (Ala-DH) leads to the recycling of alanine with ammonia. Ala-DH needs NADH as a cofactor, so a recycling system with the before mentioned FDH or GDH is required.[6]

## 1.2. Chemical oxidation and reductive amination of alcohols

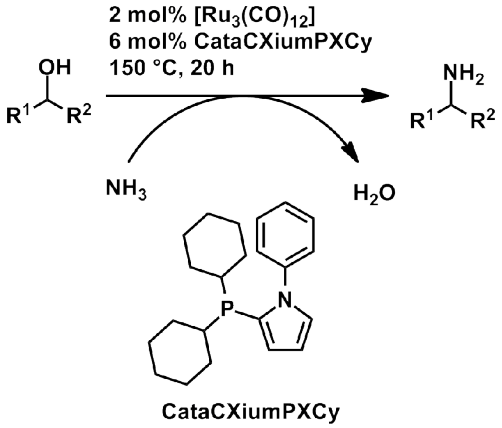
The introduction of a nitrogen atom in a highly selective way is a challenge for organic synthesis.[12] The direct amination of primary or secondary alcohols requires at least two steps, therefore a direct approach is of high interest.[13] Most methods that provide direct amination

are referred as "hydrogen shuttling".[14] These processes work with extreme conditions and are often not selective and lead to product mixtures.[15] Therefore scientific research has been focused on the invention of catalysts with improved chemoselectivity. Millstein *et al.* used a Ruthenium complex and ammonia to form primary amines (Scheme 3).[13] [16]




**Scheme 3: Ruthenium catalyst for direct amination of alcohols[13] [16]**

Another Ru catalyst [$Ru_3(CO)_{12}$] with the ligand 2-(dicyclohexylphosphino)-1-phenyl-1*H*-pyrrole (CataCXiumPCy) can aminate secondary alcohols (Scheme 4).[17] [18]




**Scheme 4: Direct amination of secondary alcohols [17] [18]**

Iridium catalysts are also able to apply ammonium as an aminating agent for alcohols.[19] The inexpensive transition metals copper and iron were also tried as catalysts. Here a substrate limitation was observed as they can only transform benzylic alcohols, whereas other substance classes are not well accepted.[20]

A Mitsunobu reaction can also be applied with the formation of an imide compound as intermediate, followed by a reduction with hydrazine.[21] [22]

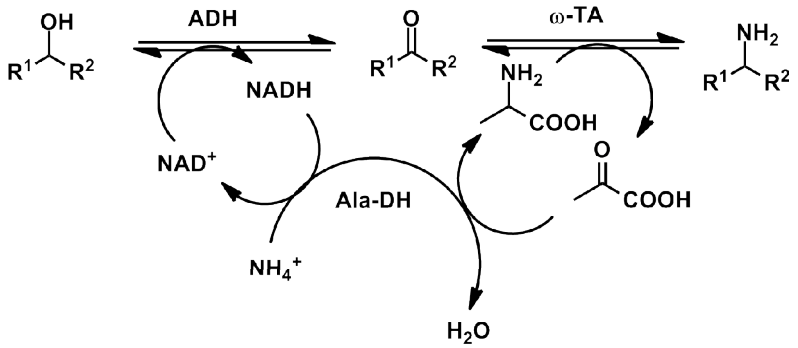
A Mitsunobu variant of the Gabriel synthesis activates the alcohol to an alkoxyphosphonium compound, which is further cleaved with HCl and forms the hydrochloride salt of the amine.[23]

## 1.3. Cascades

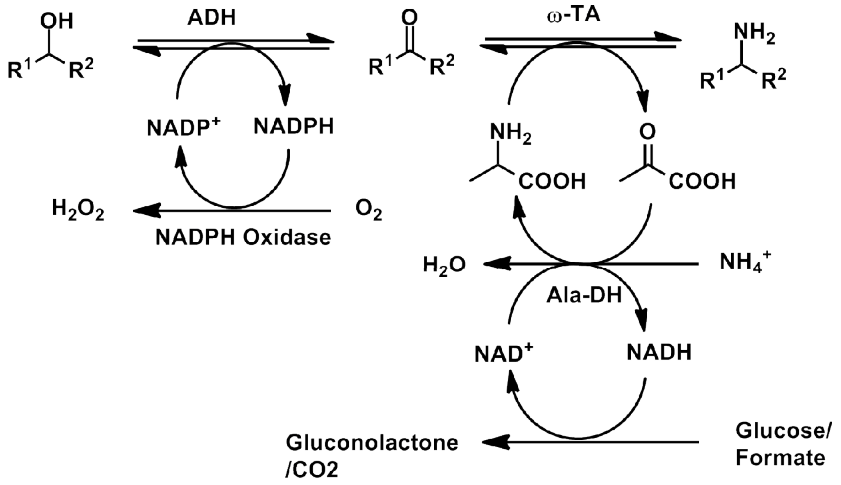
Oxidizing enzymes were combined with transaminases to form amines with alcohols as starting material. These cascade reactions avoid expensive transition metal catalysis or extreme reaction conditions. For the oxidising part of the reaction, laccases, alcohol dehydrogenases (ADHs) and oxidases can be used. For alcohol dehydrogenases a process with simultaneous reduction and oxidation has been established.[24]

When ADHs are combined with ω-TAs an internal as well as an external cofactor recycling system can be applied. (Scheme 5).[25] [26]

**a)**




**b)**




**Scheme 5: a) Internal cofactor recycling; b) Independent cofactor recycling**

The equilibrium is pushed to product formation. This is due to the fact that the carbonyl moiety is "removed" from the reaction mixture from the ω-TA *via* reductive amination. The amine donor L-alanine is recycled therefore the equilibrium in the second reaction is shifted towards the formation of the amine. This is true theoretically for primary alcohols, whereas for *sec*-alcohols the reaction reaches an equilibrium.[25] [26] Primary alcohols form a highly reactive aldehyde intermediate whereas secondary alcohols form the less reactive ketones.

Laccases need an additional redox mediator, usually a (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) derivative. To date, a one-pot cascade system has not been established.

For oxidases one-pot cascade reactions were already realised.[27] The galactose oxidase from *Fusarium* NRRL 2903 was used as oxidising enzyme and oxygen provided the electrons for the reaction.[28] [29] The inhibition of the active site of the oxidase by the recycling system for the amination step was a problem to overcome. Formate ions from the formate/FDH recycle system and high loading with the amine donor L-alanine limited the conversion. This problem was solved by the use of the glucose/GDH recycle system and a reduction of the added L-alanine.[30]

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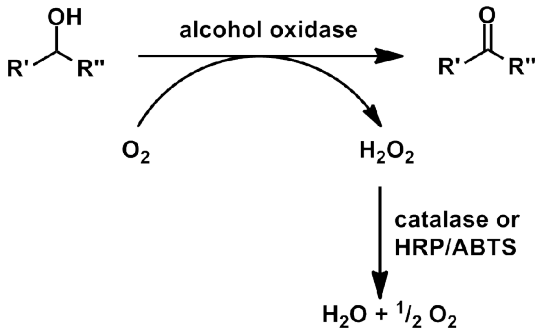
## 1.4. Oxidases

The chemical protocols used for the oxidation of alcohols apply i) toxic transition metals oxidising in stoichiometric or in catalytic amount, ii) a metal-free oxidation based on dimethyl sulfoxide (Swern) or other activating compounds or iii) molecular oxygen as oxidising reagent.[1]

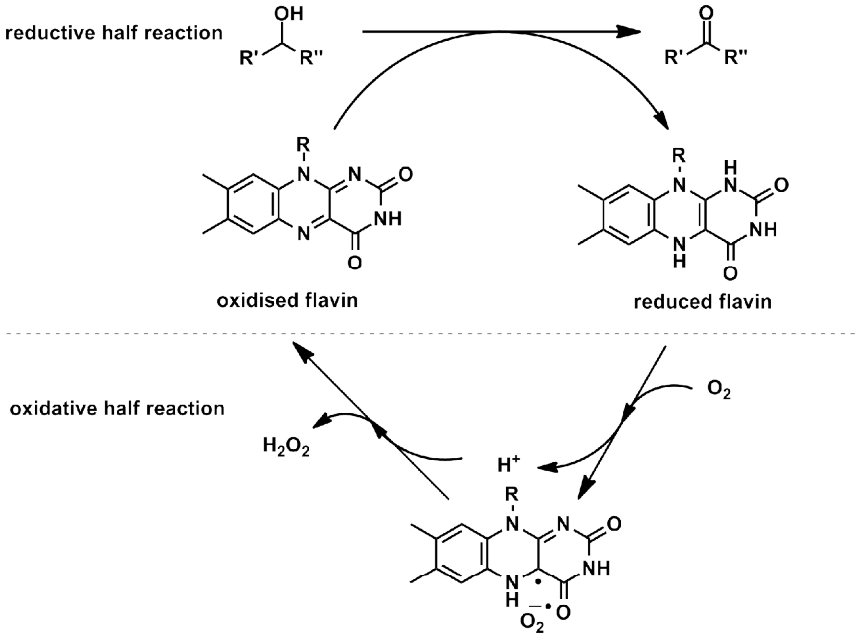
Overall, oxidation represents a fundamental reaction in nature. Oxidases, beside others, are a prominent class of enzymes able to catalyse this reaction using oxygen either as an oxidant or as an electron acceptor. They are highly active on a variety of compounds and can be used for the production of several bulk chemicals.[2] Oxidation in nature occurs with a huge variety of substrates. For example, terminal olefins are oxidised to epoxides, thioethers to the corresponding sulfoxides or primary amines to the corresponding nitroso group are oxidised by enzymes.[3] Furthermore, alcohols represent an important class of substrates for oxidases in nature as well as synthetic chemistry. Due to the high demand of oxidation products of alcohols as building blocks in many fields of chemistry, a number of biocatalytic approaches were developed using oxidases to convert primary and secondary alcohols to their corresponding aldehydes and ketones. Molecular oxygen is reduced to hydrogen peroxide while the aldehyde or keto moiety is formed (scheme 6).




**Scheme 6: Oxidation of alcohols to the corresponding carbonyl functionality using oxidases and the cleavage of hydrogen peroxide using catalase or HRP/ABTS**

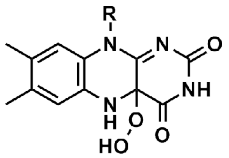
Since hydrogen peroxide may cause inactivation of the protein or lead to cell death, a catalase or a chemoenzymatic equivalent [e.g. horse radish peroxidase (HRP) and 2,2'-azino-di-(3-ethylbenzthiazolin-6-sulfonic acid (ABTS)] are often introduced in the biocatalytic system to cleave the peroxide to water and oxygen as harmless byproducts (scheme 1). ABTS shifts its absorption maximum during this process and is therefore often applied in a spectrophotometric assay.[4] An assay employing HRP, phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) was developed by Vojinović *et al.*[5]

Redox cofactor lacking oxidases are reported.[6] However, organic and metal cofactors are employed by enzymes, as the apoproteins by themselves are usually poor in mediating redox reactions. One large group of enzymes which utilise an organic cofactor are the flavoprotein oxidases. These enzymes use flavin adenine dinucleotide (FAD) or less common flavin mononucleotide (FMN) as a cofactor.[7] These enzymes are described in a review by Dijkman *et al.*[8] The oxidation is performed by two half reactions. In the reductive half reaction the alcohol substrate is oxidised by a two electron transfer. In the oxidative half reaction the oxidised flavin is regenerated by a stepwise electron transfer. The latter requires the triple state of the electrons in the oxygen molecule, as it is a spin forbidden reaction. Hence, dioxygen acts as the electron acceptor and forms a superoxide, which further oxidises the flavin and reduces oxygen to hydrogen peroxide (Scheme 7) demanding also a positive net charge.[8]



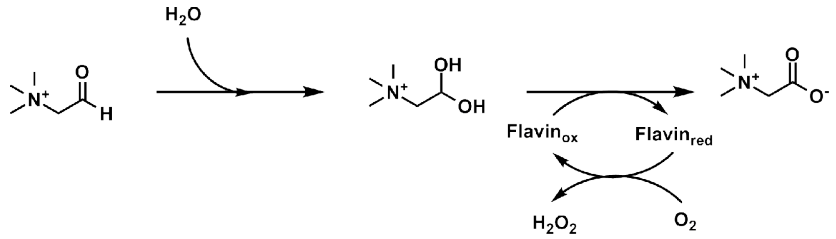

**Scheme 7: Oxidation of alcohols *via* flavin-dependent oxidase**

Up to now, the highly instable intermediate [C4a-hydroperoxyflavin (Scheme 8)] has only been detected for pyranose oxidase and is topic of further investigations.[9] [10] A mechanistic insight in the oxidation by flavin proteins has been reported by Gadda.[11]



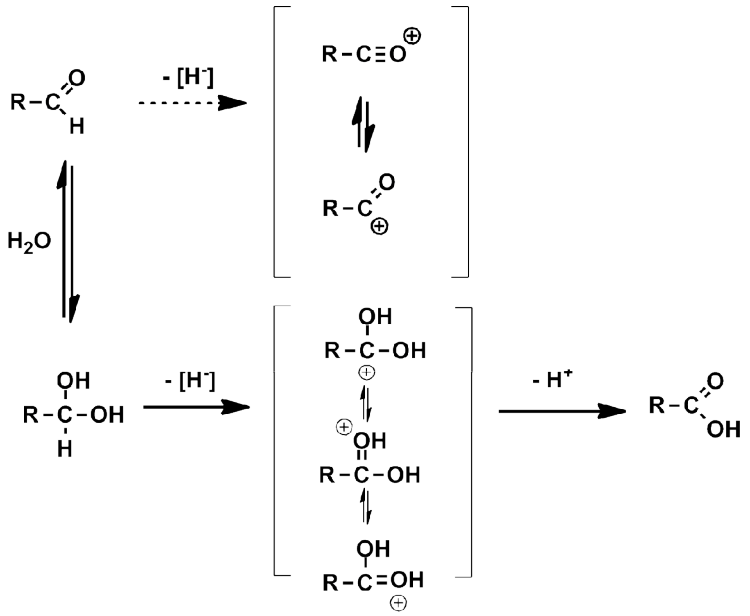
**Scheme 8: C4a-hydroperoxyflavin ï  intermediate formed by pyranose oxidase**

Oxidases do not necessarily stop their oxidation reaction at the carbonyl function as they can oxidise aldehydes to the corresponding carboxylic acids. For cholin oxidase, whose natural product is the carboxylic acid, a mechanism has been proposed. In presence of water, the geminal diol ('hydrate') of the betaine aldehyde is formed, which is subsequently oxidised to the corresponding carboxylic acid (Scheme 9).[12]



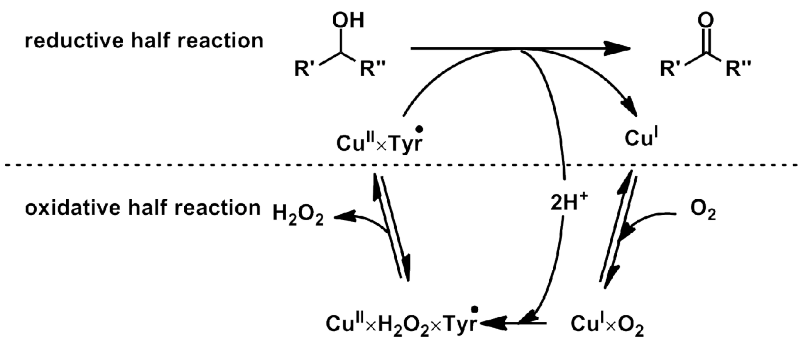
**Scheme 9: Hydration and subsequent oxidation of betaine aldehyde**

The over-oxidation to the carboxylic acid has not only been observed for cholin oxidases, but also for other flavoprotein oxidases such as alditol oxidase. Labelling studies have shown that the aldehyde hydrate occurs as intermediate.[13] This intermediate is favored in this respect, as the abstraction of a hydride from the aldehyde and with this the hypothetical acylium ion is highly unlikely (Scheme 10).




**Scheme 10: Oxidation of the aldehyde *via* its hydrate intermediate**

Another redox cofactor used by oxidases is the transition metal copper, which has been well described in several reviews.[14] [15] Since only a single copper(I) ion was found in the active site of oxidases, it seems surprising that a two-electron transfer can occur. However, it turned out that a stabilised tyrosin radical acts in the active site as a second catalytic center. The cooperative-catalytic mechanism occurs as the copper(I) just can only act *via* two-step one electron transfer, so the tyrosin radical is formed as intermediate.[16] The mechanism involves a five atom transition state and a copper(I)-tyrosine radical. In case of the copper-dependent galactose oxidase, the mechanism is well investigated.[17] The oxygen accepts the electrons, which subsequently leads to an oxidation of the primary or secondary alcohol functionality (Scheme 11). The phenomenon of over-oxidation could not be observed.[16] A detailed insight in the catalytic activation of the copper-dependent enzyme galactose oxidase was given by Whittaker.[18]




**Scheme 11: Oxidation in copper-dependent oxidases**

A broad range of oxidases have been characterised and their substrate scope was investigated. The natural substrates for these biocatalytically interesting enzymes differ quite strongly as their use in nature is diverse.[3] In fungi, oxidases were often found to be extracellular. The supplied hydrogen peroxide is used for lignin degradation by peroxidases. Furthermore, antibiotic use of the hydrogen peroxide was found in the rhizosphere, a small region of soil directly influenced by roots.[16] The investigation of the substrate scope of fungal is still ongoing. In the following, an overview of the current literature of alcohol oxidases is given, focused on the substrate tolerance.

- Primary aliphatic alcohols
- Secondary aliphatic alcohols
- Allylic alcohols
- Benzylic alcohols
- Sugar related compounds
    - Sugar alcohols and amino-sugars
    - Sugars
- Nucleosides
- Steroids
- α-Hydroxy acids
- Further activities

## 1.5. Primary aliphatic alcohols

In this section, the enzymatic oxidation of aliphatic, non activated unsaturated alcohols and 1,2-diols (where the terminal alcohol function is oxidised) and branched primary alcohols is summarised. The products of the biotransformation were the corresponding aldehydes and carboxylic acids. Which substrate was oxidised clearly depends on the enzyme. Aliphatic alcohols with a chain length of one to seven C-atoms were oxidised by short chain alcohol oxidases [SCAO EC 1.1.3.13] from several microorganisms (*Pichia pastoris*, *Hansenula* sp., *Thermoascus aurantiacus*, methanol and ethanol also alcohol oxidase from *Candida boidinii* and *Aspergillus terreus*). Here activity decreases with increasing chain length of the fatty alcohol, ranging from the highest relative activity with methanol to 24 % relative to methanol in case of 1-pentanol.[19] In a multistep oxidase/C-C-lyase reaction, short chain alcohols were oxidised by SCAO from *Hansenula* sp. (methanol, ethanol, propanol and 1-pentanol with excellent conversion or rather low for butanol) and then coupled with benzoin to form 2-hydroxyketones.[20] Alcohol substrates with a chain length of seven to sixteen carbon atoms have been best oxidised by long chain fatty alcohol oxidases [LCAO EC 1.1.3.20] from various sources (*Aspergillus terreus*, *Candida tropicalis* and *Arabidopsis thaliana*). Both, the SCAO and the LCAO, are flavin proteins

found in fungi, which were located in the microsomes.[21] [22] Terminal polar alcohols such as ω-hydroxy fatty alcohols and ω-carboxy fatty alcohols with a long hydrocarbon backbone were also oxidised by long chain alcohol oxidases.[21]

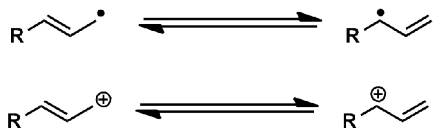
Saturated and unsaturated 1,2-diols were oxidised by alditol oxidase [EC 1.1.3.41] from *Streptomyces coelicolor*. This enzyme apparently needs a second hydroxy group in 2-position to accept the substrate, but also 1,3-butanediol was accepted as a substrate.[13] Short unsaturated alcohols without a second hydroxy group were completely (4-penten-1-ol) or partially (3-buten-1-ol) converted by short chain alcohol oxidase from *Pseudomonas* sp.[23] Furthermore, short chain alcohol oxidase from several microorganisms (*Candida boidinii, Hansenula* sp.*, Pichia pastoris, and Torulopsis methanothermo*) have been described to perform the oxidation of racemic branched alcohols in an enantioselective fashion with conversions between 16-76 % and moderate optical purities of the non-racemic substrates (*ee*s up to 40).[24] Halogen substituted alcohols, which were oxidised by SCAO were used as molecular probes for mechanistic studies.[25] For alditol oxidase (from *Streptomyces coelicolor* and *Acidothermus cellulolyticus*) it has been reported that the (*R*)-enantiomer of 1-phenyl-1,2-ethanediol carring a bulky aryl moiety was accepted.[13]

## 1.6. Secondary aliphatic alcohols

Racemic secondary aliphatic alcohols are interesting substrates for kinetic resolution.[1] The products of the oxidation were solely ketones. Several enzymes were found to be highly active towards this class of substrates. Secondary alcohol oxidase [SAO EC 1.1.3.18] from *Pseudomonas putida* or *Pseudomonas vesicularis* and *Aspergillus terreus* has shown high activity for polyvinyl alcohol (PVA).[21] [26] [27] It has been discovered that one non-heme $Fe^{2+}$ species is present in the enzyme. It is still unclear whether the iron species serves as coenzyme and if it exhibits the same role as flavin or more likely acts like the copper in galactose oxidase or takes part in the enzymatic reaction at all. For further secondary alcohols the relative activity of SAO from *Pseudomonas putida* ranges between 15-30 % (compared to PVA). Higher activity for 2-octanol has been found in the enzyme originating from *Pseudomonas vesicularis* (83 % rel. activity). Furthermore, cyclohexanol has also been accepted by the latter enzyme (42 % rel. activity), which is an interesting substrate, as its oxidised product (cyclohexanone) is used as a starting material for the synthesis of ε-caprolactam, a polymer building block. Additionally, SCAO and LCAO from *Candida tropicalis* showed broad activity on secondary alcohols.[22] [28] 2-Methyl-2-propanol was claimed to have 16 % relative activity for SCAO, but should be a non substrate due to the chemical nature of the substrate.[19]

## 1.7. Allylic alcohols

In contrast to saturated (non-activated) aliphatic alcohols, allylic and benzylic alcohols are easier to oxidise benzylic because radicals and carbene ions occurring as intermediates are resonance stabilised (Scheme 12).




**Scheme 12: resonance stabilisation of allylic species**

The oxidation of allylic alcohols was performed by the copper-dependent galactose oxidase [GOase EC 1.1.3.9] as well as by the flavoproteins cholesterol oxidase [EC 1.1.3.6] and aryl alcohol oxidase [AAO EC 1.1.3.7].[29] [30] [31] Allylic alcohol has been oxidised poorly by galactose oxidase which also accepts cinnamyl alcohol as substrate. A mutant of galactose oxidase from *Fusarium* sp. oxidised cinnamyl alcohol with full conversion.[4] Cholesterol oxidase from *Rhodococcus erythropolis* has been reported to convert several allylic alcohols in a stereo- and complete enantioselective fashion, even if the applied substrates were rather small compared to the natural substrate cholesterol. [30] Conversions up to 70 % and high to excellent *ees* were observed. For methyl-substituted bicyclic substrates the position of the hydroxyl group with respect to the methyl group was fundamental to be accepted. Aryl alcohol oxidase exhibited a broad substrate scope and accepted phenyl substituted allylic alcohols such as coniferyl and cinnamyl alcohol as well as linear molecules, such as 2,4-hexadien-1-ol.[32]

## 1.8. Benzylic alcohols

Numerous benzylic alcohols were reported to be oxidised mainly by galactose oxidase from *Fusarium* NRRL 2903 and by aryl alcohol oxidase from *Pleurotus eryngii*. In the case of benzyl alcohol two more species of AAO have shown activity (AAO from *Aspergillus terreus* and *Pleurotus ostreatus*) as well as SCAO from *Candida boidinii* and SAO from *Pseudomonas vesicularis*. Various substituents on the aromatic ring system have been accepted by the enzyme candidates. All three regioisomers of pyridine methanol were transformed by a mutant of galactose oxidase. This mutant showed up to 2000-fold increase of activity towards 2-pyridine methanol compared to the canonical D-galactose.[29] A broad scope of substituted benzyl alcohols was tested to define the catalytic reaction profile for galactose oxidase from *Fusarium. Meta*- and *para*-substituted substrates (3-F, 3-Br, 3-Cl, 3-$NO_2$, 4-F, 4-Cl, 4-I, 4-OMe, 4-SMe, 4-Me, 4-$CF_3$) were converted with up to 20-fold variation of relative rates.[33] Also the wild type of galactose

oxidase from *Fusarium* has a broad substrate scope, the activity was increased by mutants. Secondary aryl alcohols have been applied for a kinetic resolution with partly excellent *ee*s using an (*R*)-selective mutant of galactose oxidase from *Fusarium* sp. investigated by Escalettes *et al.*[34] The utilisation in a kinetic resolution process of an atropisomer has been achieved.[35]

Methoxy groups were accepted independently from the position on the ring. The relative activity of methoxy substituted substrates to the unsubstituted benzyl alcohol was similar (3-methoxybenzyl alcohol) or more than 5-fold higher for the *para*-substituted substrate. Furthermore, dimethoxy substituted alcohols were converted by aryl alcohol oxidase with high activity. The 3,4-dimethoxybenzyl alcohol, named veratryl alcohol, was converted with 326 % relative activity to benzyl alcohol whilst the 2,4-substituted pendant was accepted with 178 % relative activity. For all these substrates the corresponding aldehydes were detected.[36] The enzyme also converted 3,4,5-trimethoxybenzyl alcohol, but with rather low activity. Besides methoxy groups, also hydroxy groups, a *meta*-substituted phenoxy group, as well as combinations of hydroxy and methoxy groups were accepted. These substrates were poorly converted compared to the 3-phenoxybenzyl alcohol which was well accepted.[30] While the enzyme seems to accept sterically high demanding substrates, such as the phenoxy substituents, additional methoxy or especially hydroxy groups seem to lead to unfavourable interactions in the active site.

Piperonyl alcohol (1,3-benzodioxole-5-methanol), a building block in the epinephrine synthesis, was oxidised with full conversion by the galactose oxidase from *Fusarium* sp.[4] A broad range of chloro and fluoro substituted aryl alcohols were accepted by both aryl alcohol oxidase and galactose oxidase. Full conversions have been reported for galactose oxidase.[31] [36] The only exception, *meta*-substituted chloro benzyl alcohol, has not been converted at all. It was not reported, whether this was due to the electronic nature of the chloroatom or because of steric reasons. A substrate which is sterically demanding and well accepted is 2-naphtalene methanol. It had a relative activity of 746 % compared to the aromatic substrate with one ring.[35] In conclusion the position of the substituents and their polarity seem to play a crucial role in substrate acceptance.

## 1.9. Sugar related compounds

### 1.9.1. Sugar alcohols and amino-sugars

Several enzyme candidates were reported to oxidise sugar alcohols to the corresponding aldoses and subsequently to the aldonic acids. FAD-dependent alditol oxidase [EC 1.1.3.41] as well as copper-dependent galactose oxidase from *Fusarium* have shown great acceptance with respect to sugar alcohols. Alditol oxidase from *Streptomyces* sp. as well as the thermophilic *Acidothermus cellulolyticus* acted on several D- and even L-sugar alcohols to oxidise them to the corresponding aldoses or even further to carboxylic acids. The substrates D-galactitol, D-xylitol, D-sorbitol, D-mannitol, L-threitol and pro-chiral glycerol were tested in kinetic studies.[13] [37] [38] [39]

For the oxidation of amino-sugars, *N*-acyl-D-hexosamine oxidase [EC 1.1.3.29] from *Pseudomonas* sp. represents an appropiate biocatalytical tool. Galactose oxidase also showed that it is able to oxidise this class of substrates.[40] *N*-Acetyl-D-galactosamine was almost converted as good as the natural substrate (98-99 % rel. activity). It seems that the configuration of C-4 is in contrast to other enzymes not relevant for the substrate acceptance of *N*-acyl-D-hexosamine oxidase. Amino-sugars without an *N*-acyl function (e.g. D-glucosamine (26 % rel. activity), and D-galactosamine (81 % rel. activity) were moderate substrates. Furthermore, *N*,*N*'-diacetylchitobiose (31-49 % rel. activity) and *N*-acetylmuramic acid (44 % rel. activity) were tested to be moderate. The activities were all measured relative to the natural substrate *N*-acetyl-D-glucosamine.[41] [42]

### 1.9.2. Sugars

In this section different sugars and their derivatives are summarised. The formed products were the lactones, the keto aldoses and diketones, when a subsequent oxidation of the keto product occurred. D-Glucose is the natural substrate of glucose oxidase [GOX EC 1.1.3.4], a flavoenzyme, very well studied from *Aspergillus niger*, which displayed a very narrow substrate spectrum.[43] [44] D-Glucose was also oxidised by pyranose oxidase [P2O EC 1.1.3.10] from several different fungi (*Peniophora gigantea*, *Trametes hirsuta*, *Trametes versicolor*, *Trametes ochracea*, *Tricholoma matsutake*, *Gloeophyllum sepiarium*, *Coriolus* sp. and *Peniophora* sp.). It oxidises hydroxyl groups on the C-2 position, but also oxidation at C-3 can occur.[45] Giffhorn summarised the properties of this flavin-dependent enzyme.[46] Hexose oxidase [HOX EC 1.1.3.5] from *Chondrus crispus* is an enzyme with a fairly broad substrate scope. The oxidation of D-glucose is the catalysed reaction in nature.[47] [48] D-Galactose, the canonical substrate for the copper-dependent galactose oxidase, was oxidised by pyranose oxidase from *Peniophora gigantea* and hexose oxidase from *Hansenula polymorpha*.[34] [46] [49] [50] It is important to note that D-galactose was a rather poor substrate for pyranose oxidase. Furthermore, the configuration on C-4 played an important role in substrate acceptance. D-Allose (94 % rel. activity), D-xylose (50-100 % rel. activity) and D-mannose (only moderate rel. activity of 23 %) were all oxidised by pyranose oxidase originating from several microorganisms.[46] [51] [52] Hexose oxidase accepted D-xylose, D-

arabinose and the D-glucose containing disugars like D-lactose and D-cellobiose.[47] [53] A galactose oxidase investigated from *Fusarium* converted D-galactose containting substrates D-lactose (48 % rel. activity), lactitol, lactobionic acid and the synthetic disaccharide and laxativum D-lactulose (rel. activity 100 %). The oxidation of the galactose moiety occurred at the C-6 carbon.[22] The disugars D-melibiose, D-raffinose and D-stachyose were good substrates for galactose oxidase (68 % rel. activity for D-melibiose, up to 161 % rel. activity for D-stachyose).[40] Pyranose oxidase accepted D-trehalose (54 % rel. activity), D-gentibiose (fairly good with 51 % rel. activity) and D-maltose (rather poor with 8 % rel. activity) as substrates. The relative activities were measured respectively to the model substrates D-galactose or D-glucose.[46] [51] [52] For D-fructose, an mutated galactose oxidase from *Fusarium* seems to be a appropiate biocatalyst.[54]

Deoxy-sugars were often employed in kinetic studies to investigate the catalytic mechanism of the enzymes. 1-, 2-, 3- And 6-deoxy-D-glucose and 2-deoxy-D-galactose were used for this purpose showing full conversions. For pyranose oxidase, activity was observed for 2-deoxy-D-glucose (52 % rel. activity), whereby oxidation at carbon-3 occurs. 1-Deoxy-D-glucose was converted by pyranose oxidase (8 % rel. activity by pyranose oxidase from *Phanerochaete gigantea*, 22 % from *Coriolus versicolor* and 69 % from *Tricholoma matsutake*). The substrate 3-deoxy-D-glucose was almost as good for pyranose oxidase as the natural one. Acceptance of 6-deoxy-D-glucose was significantly worse with only 15 % relative activity. Glucose oxidase also shows activity for 1-deoxy-D-glucose and 6-deoxy-D-glucose. 2-Deoxy-D-galactose showed 74 % relative activity and 2-deoxy-D-glucose 60 % for galactose oxidase.[23] [44] [46] [51] [52] [55]

Various sugar derivatives were tested. *O*-Methylated sugars were accepted by pyranose oxidase and galactose oxidase.[23] [40] [44] [49] [52] [56] With pyranose oxidase the the oxidation occurred at the C-3 carbon atoms. Phenyl- and hexyl-glucoside substrates are well accepted, but perform a transfer reaction and form a disaccharide. These bulky substrates indicate that the size of the active site is not a limiting factor. Nitro-sugars were tested in case of pyranose oxidase (at C-2 position 15 % yield of a product mixture, at C-4 position 24 % yield of a product mixture). *α*-D-Glucosyl fluoride was a moderate substrate with 40 % overall yield employing pyranose oxidase from *Peniophora gigantea*.[46] [52] Pyranose oxidase also converted the L-sugar L-sorbose completely. Derivatives of hydroxyacetone represented excellent substrates for galactose oxidase. Furthermore, the latter enzyme was active on guaran, a galactomannan (47 % rel. activity).[40]

## 1.10. Nucleosides

Nucleoside oxidase [EC 1.1.3.28] is an enzyme which has a broad substrate scope including natural and non natural nucleosides. The products found were the corresponding nucleoside 5'-aldehydes or carboxylic acids. The enzymes which have been investigated so far originated from the microorganism *Flavobacterium meningosepticum.* Relative to adenosine, the activity regarding other nucleosides was excellent (around 90 % rel. activity). In general, the enzyme is slightly less active when 2'-deoxynucleosides were applied as substrates (between 26-66 % rel. activity). Several substituted and derivatised nucleosides have been tested as substrates with varying success. The modified 2'-tosyladenosine is a rather poor substrate with relative activity of 19 %, whereas 8-bromoadenosine (49 % rel. activity), *N*-benzoyl-2'-deoxyadenosine (64 % rel. activity) and 1,$N^6$-ethenoadenosine (56 % rel. activity) are moderate substrates. In contrast, adenosine-*N*'-oxide (90 % rel. activity) and 2'-deoxy-1-methyladenosine (92 % rel. activity) are fairly good substrates. Modified purine nucleosides were accepted with an activity up to 90 %. A range of bioactive compounds have been reported to be oxidised by nucleoside oxidase, such as the purine phosphorylase inhibitors Tubercidine (100 % rel. activity) and Formycin B (76 % rel. activity), the proapoptotic antiproliferate plant growth regulator Kinetine riboside (89 % rel. activity) and the antiviral agent Ribavirin (14 % rel. activity). Relative activities have been expressed relative to the model substrate adenosine.[57]

## 1.11. Steroids

Cholesterol oxidase [EC 1.1.3.6] found in *Streptomyces hygroscopicus*, *Rhodococcus* and *Brevibacterium sterolicum* and ecdysone oxidase [EC 1.1.3.16] from *Calliphora vicina* have been reported to oxidise steroids.[58] The flavoprotein cholesterol oxidase not only oxidises the alcohol functionality, but also isomerises the double bond between carbon 5 and 6 into conjugation with the keto function. However, the double bound was no necessity for the substrate acceptance. Hence, 5-cholesten-3-one is the product formed by cholesterol oxidase. The mechanism was found to act either in a ping-pong or sequential fashion.[59] The enzyme exhibited a surprisingly broad and unexpected substrate scope. Biellmann detected the lack of enantiospecificity for cholesterol oxidase from *Rhodococcus erythropolis*.[60] For cholesterol oxidase from *Streptomyces* sp., a number of mutants were investigated. For the enzyme from *Rhodococcus* sp. moderate activities on $\beta$-sitosterol (80 % rel. activity) and stigmasterol (78 % rel. activity) were found by Wang *et al.* Furthermore, the enzyme was active on *trans*-dehydroandrosterone (15-37 % rel. activity), cholestanol and 7-dehydrocholesterol and 5 % relative activity was found on 5$\alpha$-androstan-3$\alpha$,17$\beta$-diol.[61] The relative activities were expressed relative to the natural substrate cholesterol. [62]

In the case of ecdysone oxidase, ecdysterone is almost accepted as well as ecdysone (95 % rel. activity). The 2-deoxy analogues are well accepted substrates for the enzyme. 2-Deoxyecdysterone was an even better substrate (108 % rel. activity) than ecdysone itself. Inokosterone (136 % rel. activity) and makisterone (134 % rel. activity) were well accepted.[59] The relative activities were expressed relative to ecdysone.

## 1.12. α-Hydroxy acids

Racemic α-hydroxy acids are especially relevant substrates due to the ability to undergo kinetic resolution.[3] The enzymes form the corresponding keto acids, which are not easily synthesised by conventional methods due to their tendency to undergo decarboxylation. A broad range of α-hydroxy acids have already been studied for their capability to act as a substrate for the FNM depending glycolate oxidase or (*S*)-2-hydroxy acid oxidase [HAOX EC 1.1.3.15] from *Spinacia oleracea*. The natural substrate is glycolic acid and the products formed are the corresponding α-keto acids, respectively. Short and medium chain 2-hydroxy acids, unsaturated *cis*- and *trans*-2-hydroxydec-4-enoic acid, the bulky phenyllactic acid and the oxygen carrying 2-hydroxy-4-pentoxybutyric acid were all substrates established using a kinetic resolution (good to excellent *ee*s). Furthermore, 3-chlorolactic acid (110 % rel. activity), 2-hydroxybutanoic acid (120 % rel. activity), 3-indolelactic acid (rather poor with 18 % rel. activity), 3,3,3-trifluorolactic acid (rather poor with 11 % rel. activity) and 2-hydroxydecanoic acid (40 % rel. activity) were examined. The relative activities were expressed relative to lactic acid. [63] [64] [65] In the case of FMN-dependent lactate oxidase [EC 1.1.3.2] from *Aerococcus vriridans* and a corresponding mutant, also more sterically demanding hydroxy acids than lactate were used as substrates as shown for several *para*-substituted mandelic acid derivatives in an quantitative-structure-analysis.[66] Additionally, an enzyme originating from *Pseudomonas stutzeri* was used to oxidise lactic acid to pyruvate enantioselectively.[67]

## 1.13. Further activities from oxidases

The biocatalytic activities of several other oxidases employing alcohols were explored. Either they exhibited a narrow substrate scope or they were only recently discovered and need more investigations, to explore their biocatalytic potential. The flavoenzyme thiamine oxidase [EC 1.1.3.23] converted thiamine to the corresponding aldehyde and even further to the carboxylic acid.[68] α-Glycerophosphate oxidase [EC 1.1.3.21] studied from *Streptococcus* sp. and *Enterococcus casseliflavus* is a FAD-dependent enzyme which converted glycerophosphate to the corresponding aldehyde.[69] The lignin degrading cellobiose oxidase [EC 1.1.3.25] a haemo-flavoprotein, was used in dye decolorising processes combined with laccase.[70] Isoamyl oxidase

[EC 1.1.3.x] from *Aspergillus oryzae* was characterised and active regarding isoamyl alcohol, while the activity for short chain alcohols was tested unsuccessfully.[71] Choline oxidase [EC 1.1.3.17] from *Arthrobacter globiformis* was the object of mechanistic studies by Gadda *et al.* [11] [12] [72] Pyridoxine-4 oxidase [EC 1.1.3.12] is a flavin-dependent enzyme which oxidises pyridoxine to the corresponding aldehyde pyridoxal in the degrading pathway of vitamin B6. This enzyme has been found in several microorganisms.[73] [74] [75]

# 2. Tables

## 2.1. Primary aliphatic alcohols

| Substrate | Oxidase | Reference |
|---|---|---|
| | mono-alcohols | |
| methanol<br>ethanol<br>1-propanol<br>1-butanol | **Short chain alcohol oxidase**[b] from *P. pastoris, Hansenula* sp. and *T. aurantiacus,* methanol and ethanol also from *C. boidinii* and *A. terreus* | [19] [20] [21] [23] [25] [76] [77] |
| 1-pentanol | **Short chain alcohol oxidase**[b] from *T. aurentiacus* and *Hansenula* sp. | [19] [20] |
| 1-heptanol | **Short chain alcohol oxidase** [b] from *A. terreus*; **Long chain alcohol oxidase** [b] from *A. terreus* | [28] [78] |
| 1-octanol | **Long chain alcohol oxidase** [b] from *A. terreus* and *C. tropicalis* | [21] [22] |
| 1-nonanol | **Long chain alcohol oxidase** [b] from *A. terreus* | [21] |
| 1-decanol | **Long chain alcohol oxidase** [b] from *A. terreus* and *C. tropicalis* | [21] [22] |
| 1-undecanol | **Long chain alcohol oxidase** [b] from *A. terreus* | [21] |
| 1-dodecanol | **Long chain alcohol oxidase** [b] from *A. terreus, A. thaliana* and *C. tropicalis* | [21] [22] [78] [79] |
| 1-tridecanol | **Long chain alcohol oxidase** [b] from *A. terreus* | [21] |
| 1-tetradecanol | **Long chain alcohol oxidase** [b] from *A. terreus* and *C. tropicalis* | [21][22] |
| 1-hexadecanol | **Long chain alcohol oxidase** [b] from *A. terreus* and *A. thaliana* | [21] [79] |
| 2-bromethanol<br>2-chlorethanol | **Short chain alcohol oxidase** from *C. boidinii* | [25] |
| | diols and triols | |
| 1,2-ethanediol | **Short chain alcohol oxidase** [b] from *T.aurentiacus* and *P. pastoris* | [19] [77] |
| 1,10-decanediol | **Long chain alcohol oxidase**[b] from *C. tropicalis* | [22] |
| 1,16-hexadecanediol | **Long chain alcohol oxidase**[b] from *A. terreus, C. tropicalis and A. thaliana* | [21] [22] [79] |
| 1,2-butanediol<br>1,2-pentanediol<br>1,2-hexanediol | **Alditol oxidase**[b] from *S. coelicolor* | [13] |
| 1,3,5-pentanetriol<br>1,2,4-butanetriol | **Alditol oxidase**[b] from *S. coelicolor* | [13] |
| 1,3-butanediol | **Alditol oxidase**[b] from *S. coelicolor* | [13] |
| 3-chloro-1,2-propanediol<br>3-bromo-1,2-propanediol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [80] |
| 1-phenyl-1,2-ethanediol | **Alditol oxidase**[b] from *S. coelicolor* and *A. cellulolyticus* 11B | [13] [39] |
| 12-hydroxydodecanoic acid | **Long chain alcohol oxidase**[b] from *A. terreus* and *C. tropicalis* | [21] [22] |
| 16-hydroxyhexadecanoic acid | **Long chain alcohol oxidase**[b] from *C. tropicalis* | [79] |
| | **unsaturated** | |
| 3-buten-1-ol<br>4-penten-1-ol | **Short chain alcohol oxidase**[b] from *P. pastoris* | [23] |
| 3-buten-1,2-diol | **Alditol oxidase**[b] from *S. coelicolor* | [13] |

| | | |
|---|---|---|
| 4-pentene-1,2-diol | | |
| | **branched** | |
| 2-methyl-1-butanol | **Short chain alcohol oxidase**[b] from *C. boidinii, P. pastoris* and *Hansenula* sp. | [24] |
| 3-methyl-1-butanol | **Short chain alchol oxidase**[b] from *T. aurentiacus* | [19] |
| 2-methyl-1-pentanol | **Short chain alcohol oxidase**[b] from *C. boidinii, P. pastoris* and *Hansenula* sp. | [24] |
| 2-methyl-1-hexanol | **Short chain alcohol oxidase**[b] from *C. boidinii, P. pastoris* and *Hansenula* sp. | [24] |
| 3-methylbutan-1-ol | **Secondary alcohol oxidase**[c] from *A. terreus* | [22] |
| 1-phenyl-3-propanol | **Short chain alcohol oxidase**[b] from *A. terreus* | [28] |

[a] Copper-dependent; [b] flavin-dependent; [c] $Fe^{2+}$-dependent

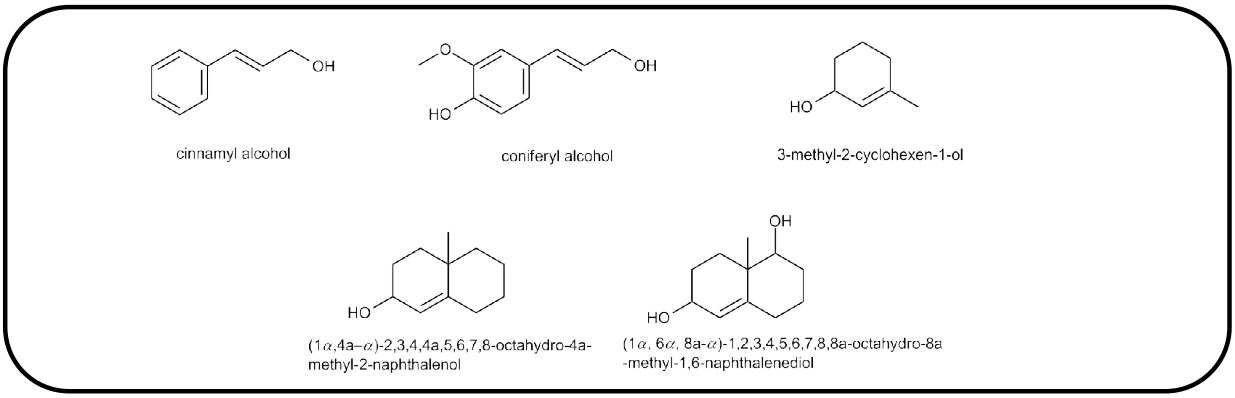
## 2.2. Secondary aliphatic alcohols

| Substrate | Oxidase | Reference |
|---|---|---|
| polyvinyl alcohol | **Secondary alcohol oxidase**[c] from *P. putida* and *P. vesicularis* | [26] [27] |
| 2-propanol | **Secondary alcohol oxidase**[c] from *A. terreus;* **Short chain alcohol oxidase**[b] from *T. aurentiacus* and *P. pastoris* | [19] [21] [77] |
| 2-butanol | **Secondary alcohol oxidase**[c] from *P. putida* and *P. vesicularis*; **Short chain alcohol oxidase**[b] from *T. aurentiacus* | [19] [26] [27] |
| 2-pentanol | **Secondary alcohol oxidase**[c] from *P. putida* and *P. vesicularis* | [26] [27] |
| 3-pentanol | **Secondary alcohol oxidase**[c] from *P. putida* | [26] |
| 2-hexanol<br>3-hexanol | **Secondary alcohol oxidase**[c] from *P. putida* and *P. vesicularis* | [26] [27] |
| 2-heptanol<br>3-heptanol<br>4-heptanol | **Secondary alcohol oxidase**[c] from *P. putida* and *P. vesicularis* | [26] [27] |
| 2-octanol | **Secondary alcohol oxidase**[c] from *A. terreus* and *P. vesicularis;* **Short chain alcohol oxidase**[b] from *A. terreus* | [21] [27] [28] |
| 3-octanol | **Secondary alcohol oxidase**[c] from *A. terreus* and *P. putida* | [22] [26] |
| 4-octanol | **Secondary alcohol oxidase**[c] from *P. putida* | [26] |
| 4-nonanol<br>5-nonanol | **Secondary alcohol oxidase**[c] from *P. putida* | [26] |
| 2-dodecanol | **Secondary alcohol oxidase**[c] from *A. terreus* | [21] |
| cyclohexanol | **Secondary alcohol oxidase**[c] from *P. vesicularis* | [27] |
| cyclooctanol | **Secondary alcohol oxidase**[c] from *A. terreus* | [21] |

| | | |
|---|---|---|
| 2-decanol | **Long chain alcohol oxidase**[b] from *C. tropicalis* | [22] |
| 4-decanol | **Secondary alcohol oxidase**[c] from *P. vesicularis* | [27] |
| 2-undecanol | **Long chain alcohol oxidase**[b] from *C. tropicalis* | |
| 2-dodecanol | | [22] |
| 2-hexadecanol | | |
| 1,2-propanediol | **Secondary alcohol oxidase**[c] from *P. putida* | [26] |
| 2,4-pentanediol | **Secondary alcohol oxidase**[b] from *P. vesicularis* | [27] |

[a] Copper-dependent; [b] flavin-dependent; [c] $Fe^{2+}$-dependent

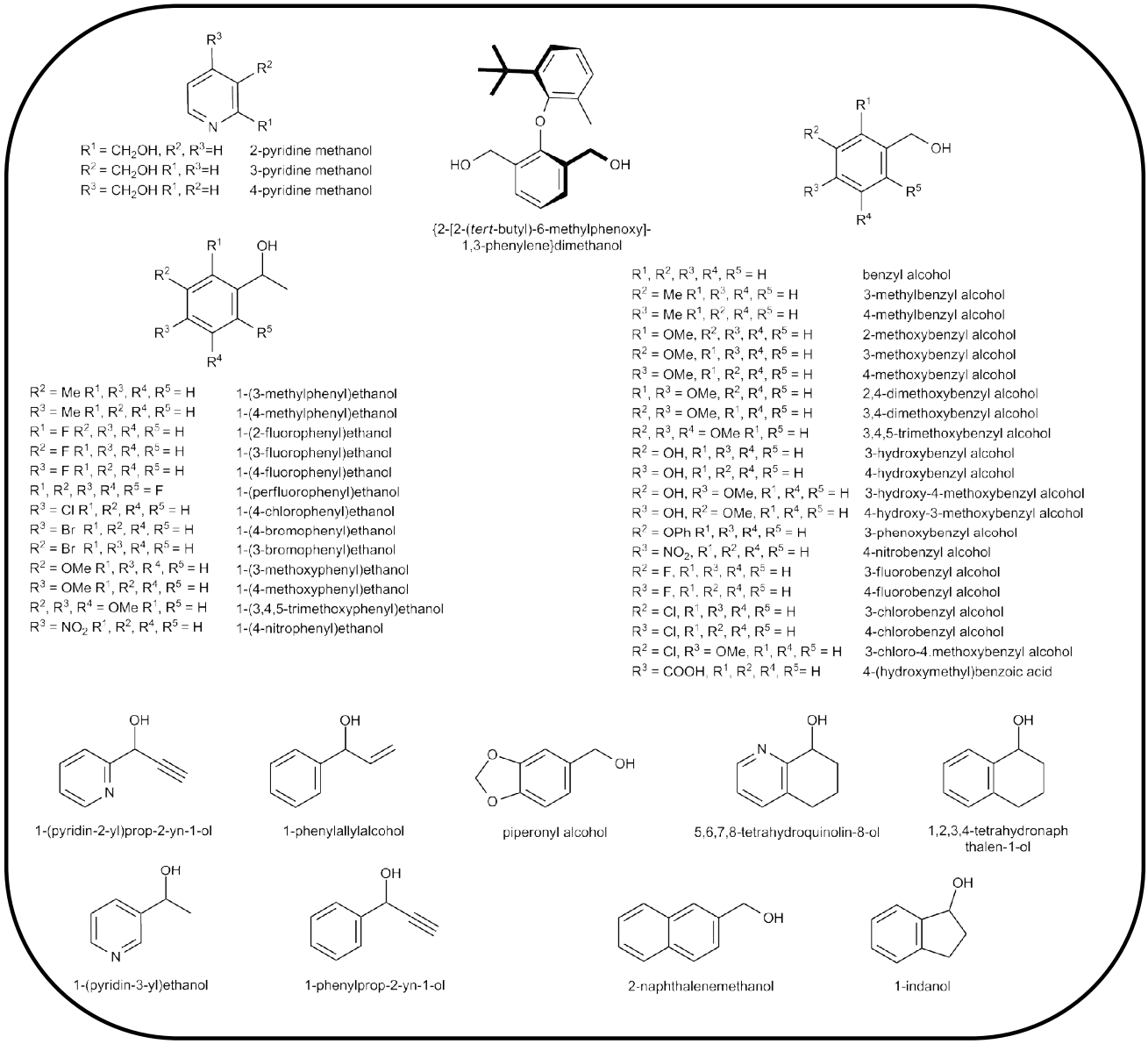
## 2.3. Allylic alcohols




| Substrate | Oxidase | Reference |
|---|---|---|
| allyl alcohol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903<br>**Aryl alcohol oxidase**[b] from *P. eryngii* | [29] [31] [40] |
| 3-buten-2-ol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [29] |
| 2,4-hexadien-1-ol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] [32] |
| cinnamyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii*;<br>**Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [4] [31] [32] [36] |
| coniferyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] |
| 3-methyl-2-cyclohexen-1-ol | **Cholesterol oxidase**[b] from *R. erythropolis* | [30] |
| (1*α*,4a-*α*)-2,3,4,4a,5,6,7,8-octahydro-4a-methyl-2-naphthalenol | **Cholesterol oxidase**[b] from *R. erythropolis* | [30] |
| (1*α*,6*α*,8a-*α*)-1,2,3,4,6,7,8,8a-octahydro-8a-methyl-1,6-naphthalenediol | **Cholesterol oxidase**[b] from *R. erythropolis* | [30] |

[a] Copper-dependent; [b] flavin-dependent

## 2.4. Benzylic alcohols



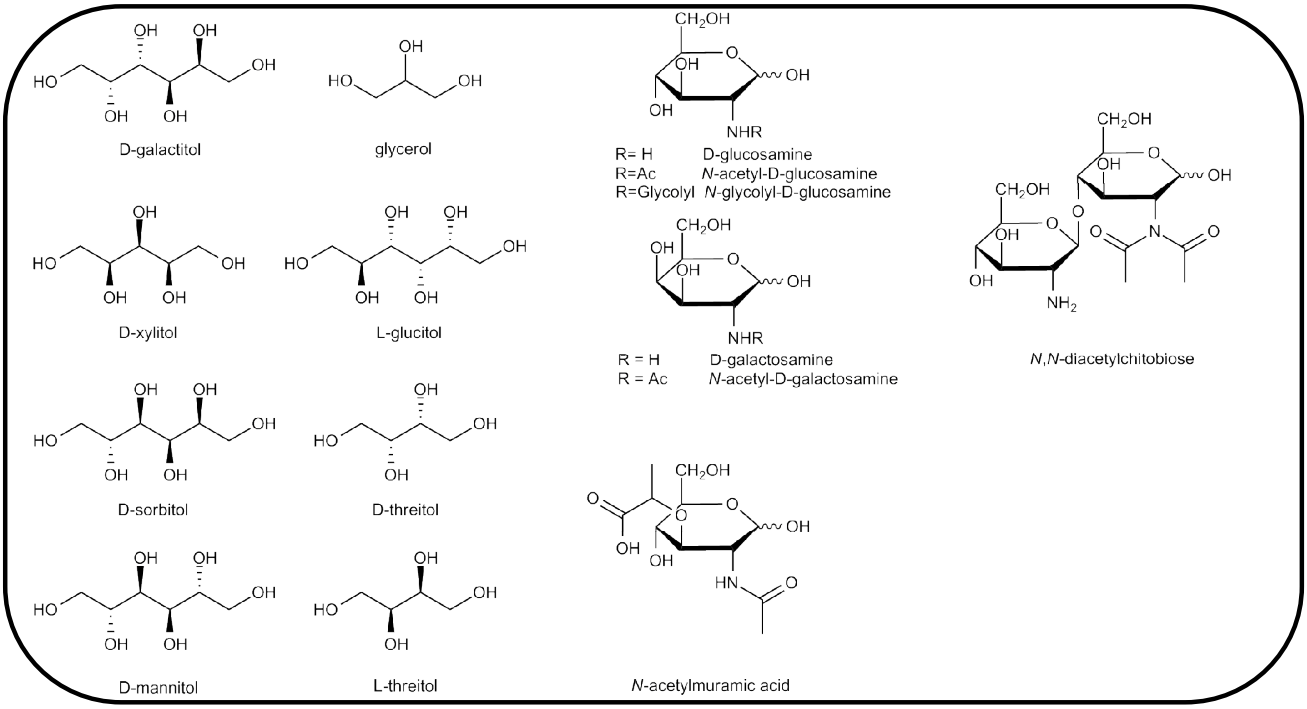

| Substrate | Oxidase | Reference |
|---|---|---|
| 2-pyridine methanol<br>3-pyridine methanol<br>4-pyridine methanol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [29] |
| benzyl alcohol | **Short chain alcohol oxidase**[b] from *C. boidini;* **Galactose oxidase**[a] *from Fusarium* NRRL 2903*;* **Aryl alcohol oxidase**[b] from *P. eryngii, A. terreus* and *P. ostreatus;* **Secondary alcohol oxidase**[c] from *P. vesicularis* | [4] [19] [25] [26] [27] [31] [32] [33] [36] [81] |
| 3-methylbenzyl alcohol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [4] [33] |

| | | |
|---|---|---|
| 4-methylbenzyl alcohol<br>2-methoxybenzyl alcohol<br>3-methoxybenzyl alcohol<br>4-methoxybenzyl alcohol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903; **Aryl alcohol oxidase**[b] from *P. eryngii,* 4-methoxybenzyl alcohol also from *P. ostreatus* | [4] [9] [31] [32] [36] [77] [82] |
| 2,4-dimethoxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [9] [31] |
| 3,4-dimethoxybenzyl alcohol | **Short chain alcohol oxidase** from *T. aurentiacus;* **Aryl alcohol oxidase**[b] from *P. eryngii* and *P. ostreatus* | [19] [31] [36] [82] |
| 3,4,5-trimethoxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] |
| piperonyl alcohol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [4] |
| 3-hydroxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] |
| 4-hydroxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] |
| 3-hydroxy-4-methoxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] [32] |
| 4-hydroxy-3-methoxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii;* **Vanillyl alcohol oxidase**[b] from *P. simplicissimum* | [31] [83] |
| 3-phenoxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] |
| 3-nitrobenzyl alcohol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [33] |
| 4-nitrobenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii;* **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [31] [33] |
| 3-fluorobenzyl alcohol<br>4-fluorobenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii;* **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [32] [33] |
| 3-chlorobenzyl alcohol<br>4-chlorobenzyl alcohol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903; 3-chlorobenzyl alcohol also from **Aryl alcohol oxidase**[b] from *P. eryngii* | [4] [32] |
| 3-chloro-4-methoxybenzyl alcohol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [32] |
| 2-naphthalenemethanol | **Aryl alcohol oxidase**[b] from *P. eryngii* | [31] [36] |
| 4-(hydroxymethyl)benzoic acid | **Aryl alcohol oxidase**[b] from *P. eryngii* | [32] |
| 1-phenylethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-(4-methylphenyl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-(2-fluorophenyl)ethanol<br>1-(3-fluorophenyl)ethanol<br>1-(4-fluorophenyl)ethanol<br>1-(perfluorophenyl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-(4-chlorophenyl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-(3-bromophenyl)ethanol<br>1-(4-bromophenyl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-(3-methoxyphenyl)ethanol<br>1-(4-methoxyphenyl)ethanol<br>1-(3,4,5-trimethoxyphenyl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-(4-nitrophenyl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-phenylallyl alcohol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-phenylprop-2-yn-1-ol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1,2,3,4-tetrahydronaphthalen-1-ol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 1-indanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |

| | | |
|---|---|---|
| 1-(pyridin-2-yl)ethanol<br>1-(pyridin-4-yl)ethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| 5,6,7,8-tetrahydroquinolin-8-ol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [34] |
| {2-[2-(*tert*-butyl)-6-methylphenoxy]-1,3-phenylene}-dimethanol | **Galactose oxidase**[b] from *Fusarium* NRRL 2903 | [35] |

[a]Copper-dependent; [b] flavin-dependent; [c] $Fe^{2+}$-dependent

## 2.5. Sugar alcohols and amino-sugars




| Substrate | Oxidase | Reference |
|---|---|---|
| D-galactitol | **Alditol Oxidase**[b] from *Streptomyces* sp.; **Galactose oxidase**[a] *from Fusarium* NRRL 2903 | [37] [84] |
| D-xylitol | **Alditol Oxidase**[b] from *Streptomyces* sp. and *A. cellulolyticus;* **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [13] [37] [39] |
| D-sorbitol | **Alditol Oxidase**[b] from *Streptomyces* sp. and *A. cellulolyticus* | [13] [37] [39] |
| D-mannitol | **Alditol Oxidase**[b] from *Streptomyces* sp. and *A. cellulolyticus* | [13] [39] |
| glycerol | **Alditol oxidase**[b] from *A. cellulolyticus*; **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [39] [85] |
| L-glucitol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [84] |
| D-threitol | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [84] |
| L-threitol | **Alditol oxidase**[b] from *S. coelicolor* | [37] |

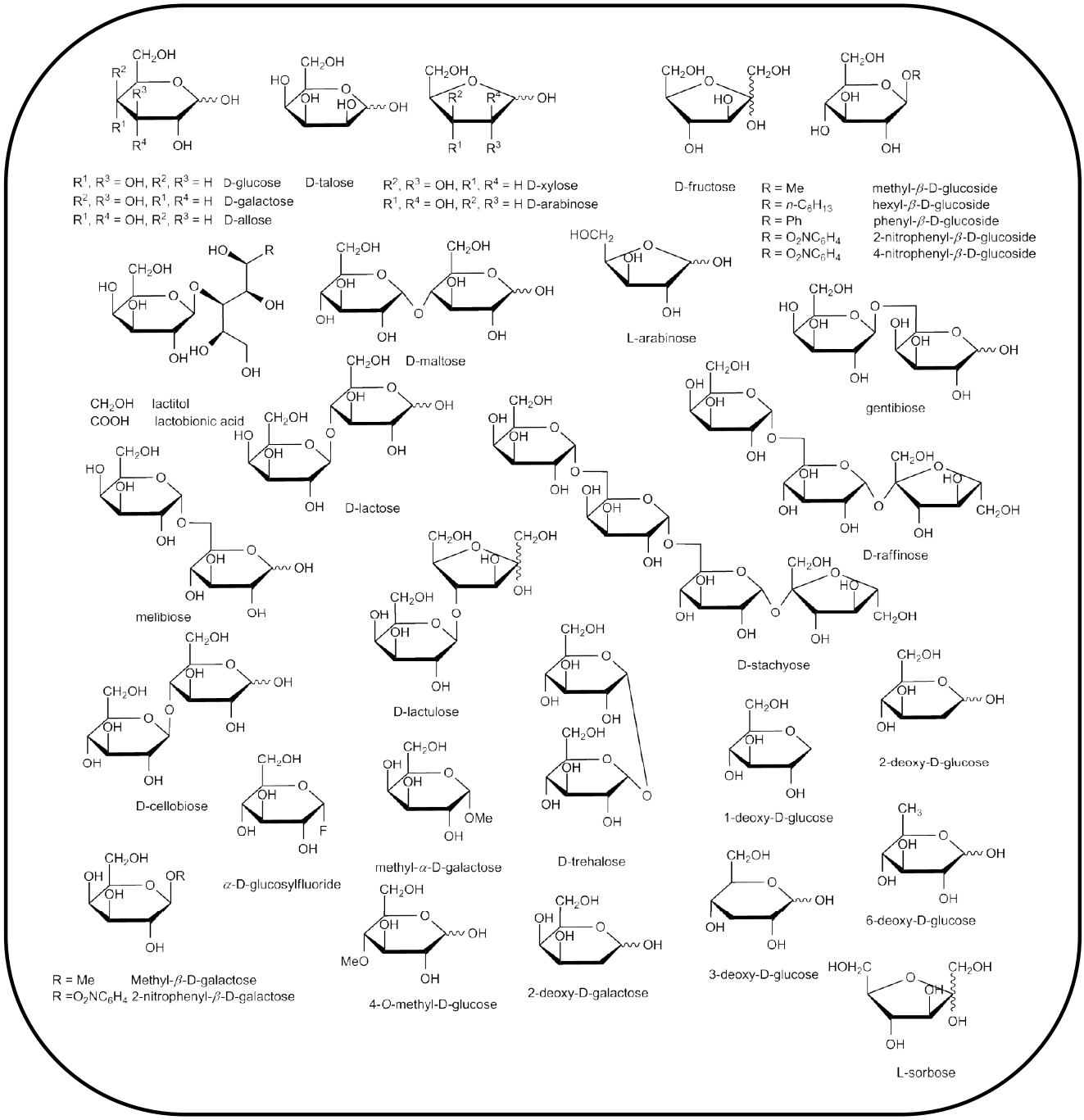
[a] Copper-dependent; [b] flavin-dependent

| Substrate | Oxidase | Reference |
|---|---|---|
| D-glucosamine<br>D-galactosamine | ***N*-acyl-D-hexosamine oxidase**[b] from *Pseudomonas* sp. D-galactosamine also from **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [40] [41] [42] |
| *N*-acetyl-D-glucosamine<br>*N*-acetyl-D-galactosamine | ***N*-acyl-D-hexosamine oxidase**[b] from *Pseudomonas* sp. | [41] [42] |

| | | |
|---|---|---|
| *N*-glycolyl-D-glucosamine | ***N*-acyl-D-hexosamine oxidase**[b] from *Pseudomonas* sp. | [41] |
| *N*-acetylmuramic acid | ***N*-acyl-D-hexosamine oxidase**[b] from *Pseudomonas* sp. | [41] |
| *N,N'*-diacetylchitobiose | ***N*-acyl-D-hexosamine oxidase**[b] from *Pseudomonas* sp. | [41] [42] |

[a] Copper-dependent; [b] probably flavin-dependent

## 2.6. Sugars




| Substrate | Oxidase | Reference |
|---|---|---|
| | **mono-sugars** | |
| D-glucose | **Glucose oxidase**[b] from *A. niger*; **Pyranose oxidase**[b] from *P. gigantea*, *T. hirsutus*, *T. versicolor*, *T. ochracea*, *T. matsutake*, | [43] [44] [46] [47] [49] [50] |

| | | |
|---|---|---|
| | *G. sepiarium*, *Coriolus* sp. and *Peniophora* sp.; **Hexose oxidase[b]** from *C. crispus* | [51] [52] [86] [87] [88] [89] [90] |
| D-galactose | **Pyranose oxidase[b]** from *P. gigantea, T. ochracea, T. matsutake;* **Galactose oxidase[a]** from *Fusarium* NRRL 2903; **Hexose oxidase[b]** from *C. crispus* | [23] [34] [40] [46] [47] [49] [50] [51] [52] [86] [88] |
| D-allose | **Pyranose oxidase[b]** from *P. gigantea* and *T. ochracea* | [46] [49] [86] [90] |
| D-xylose | **Pyranose oxidase[b]** from *P. gigantea, C. versicolor, T. ochracea* and *T. matsutake*; **Hexose oxidase[b]** from *C. crispus* | [46] [49] [51] [52] [53] [89] [90] |
| D-arabinose | **Hexose oxidase[b]** from *C. crispus;* **Pyranose oxidase[b]** from *T. matsutake* | [50] [51] [53] |
| D-mannose | **Pyranose oxidase[b]** from *T. matsutake* | [51] |
| D-fructose | **Galactose oxidase[a]** from *Fusarium* sp. | [54] |
| lactitol<br>lactobionic acid | **Galactose oxidase[a]** from *Fusarium* NRRL 2903 | [23] |

**oligosugars**

| | | |
|---|---|---|
| D-maltose | **Pyranose oxidase[b]** from *P. gigantea* and *T. matsutake;* **Hexose oxidase[b]** from *C. crispus;* | [47] [50] 51] [52] |
| D-lactose | **Galactose oxidase[a]** from *Fusarium* NRRL 2903*;* **Hexose oxidase[b]** from *C. crispus;* | [23] [40] [47] [50] [55] |
| D-gentibiose | **Pyranose oxidase[b]** from *P. gigantea* | [46] [86] |
| D-melibiose | **Galactose oxidase[a]** from *Fusarium* NRRL 2903 | [23] [40] |
| D-raffinose | **Galactose oxidase[a]** from *Fusarium* NRRL 2903 | [23] [40] [55] |
| D-stachyose | **Galactose oxidase[a]** from *Fusarium* NRRL 2903 | [40] |
| D-lactulose | **Galactose oxidase[a]** from *Fusarium* NRRL 2903 | [23] |
| D-cellobiose | **Hexose oxidase[b]** from *C. crispus* | [47] [50] |
| D-trehalose | **Pyranose oxidase[b]** from *T. matsutake* | [51] |

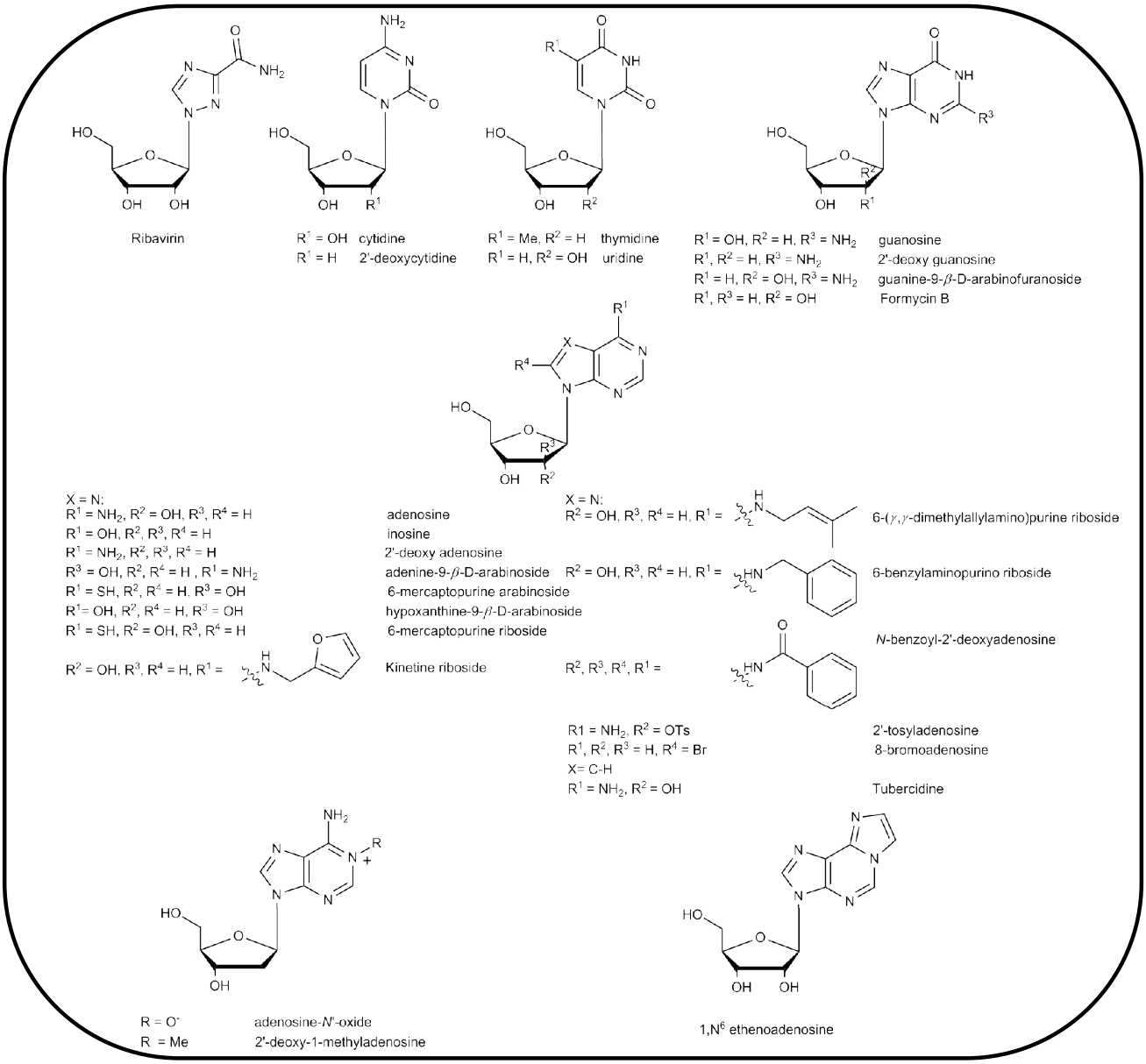
**deoxy-sugars**

| | | |
|---|---|---|
| 1-deoxy-D-glucose | **Pyranose oxidase[b]** from *P. gigantea, C. versicolor, T. matsutake* | [46] [51] [82] [85] |
| 2-deoxy-D-glucose | **Glucose oxidase[b]** from *A. niger*; **Galactose oxidase[a]** from *Fusarium* NRRL 2903; **Pyranose oxidase[b]** from *P. gigantea* and *T. ochracea* | [40] [43] [44] [45] [46] [49] [86] |
| 3-deoxy-D-glucose | **Pyranose oxidase[b]** from *P. gigantea* | [46] [86] |
| 6-deoxy-D-glucose | **Pyranose oxidase[b]** from *P. gigantea;* **Glucose oxidase[b]** from *A. niger* | [44] [46] [86] |

| | | |
|---|---|---|
| 2-deoxy-D-galactose | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [23] [40] |
| | **derivatised sugars** | |
| methyl-*β*-D-glucoside | **Pyranose oxidase**[b] from *P. gigantea* and *T. ochracea* | [45] [46] [49] [52] [86] |
| methyl-*α*-D-galactoside | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [23] [40] [55] |
| methyl-*β*-D-galactoside | **Galactose oxidase**[a] from *Fusarium* NRRL 2903; **Pyranose oxidase**[b] from *P. gigantea* and *T. ochracea* | [23] [45] [46] [55] |
| 4-*O*-methyl-D-glucose | **Glucose oxidase**[b] from *A. niger* | [44] |
| hexyl-*β*-D-glucoside<br>phenyl-*β*-D-glucoside | **Pyranose oxidase**[b] from *P. gigantea* | [46] |
| *O*-nitrophenyl-*β*-D-galactoside | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [40] |
| 2-nitrophenyl-*β*-D-glucoside<br>4-nitrophenyl-*β*-D-glucoside | **Pyranose oxidase**[b] from *P. gigantea* | [46] |
| *α*-D-glucosylfluoride | **Pyranose oxidase**[b] from *P. gigantea* | [46] |
| 2-fluoro-2-deoxy-D-glucose | **Pyranose oxidase**[b] from *P. gigantea* | [45] |
| | **L-sugars** | |
| L-sorbose | **Pyranose oxidase**[b] from *P. gigantea, C. versicolor, T. ochracea, T. matsutake* and *Peniophora* sp. | [46] [49] [52] [51] [89] [90] |
| L-arabinose | **Alditol oxidase**[b] from *S. coelicolor* | [12] |
| 1,3-dihydroxypropanone | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [23] [40] |
| 3-hydroxy-2-oxopropyl phosphate | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [85] |
| guaran | **Galactose oxidase**[a] from *Fusarium* NRRL 2903 | [40] |

[a] Copper-dependent; [b] flavin-dependent

## 2.7. Nucleosides

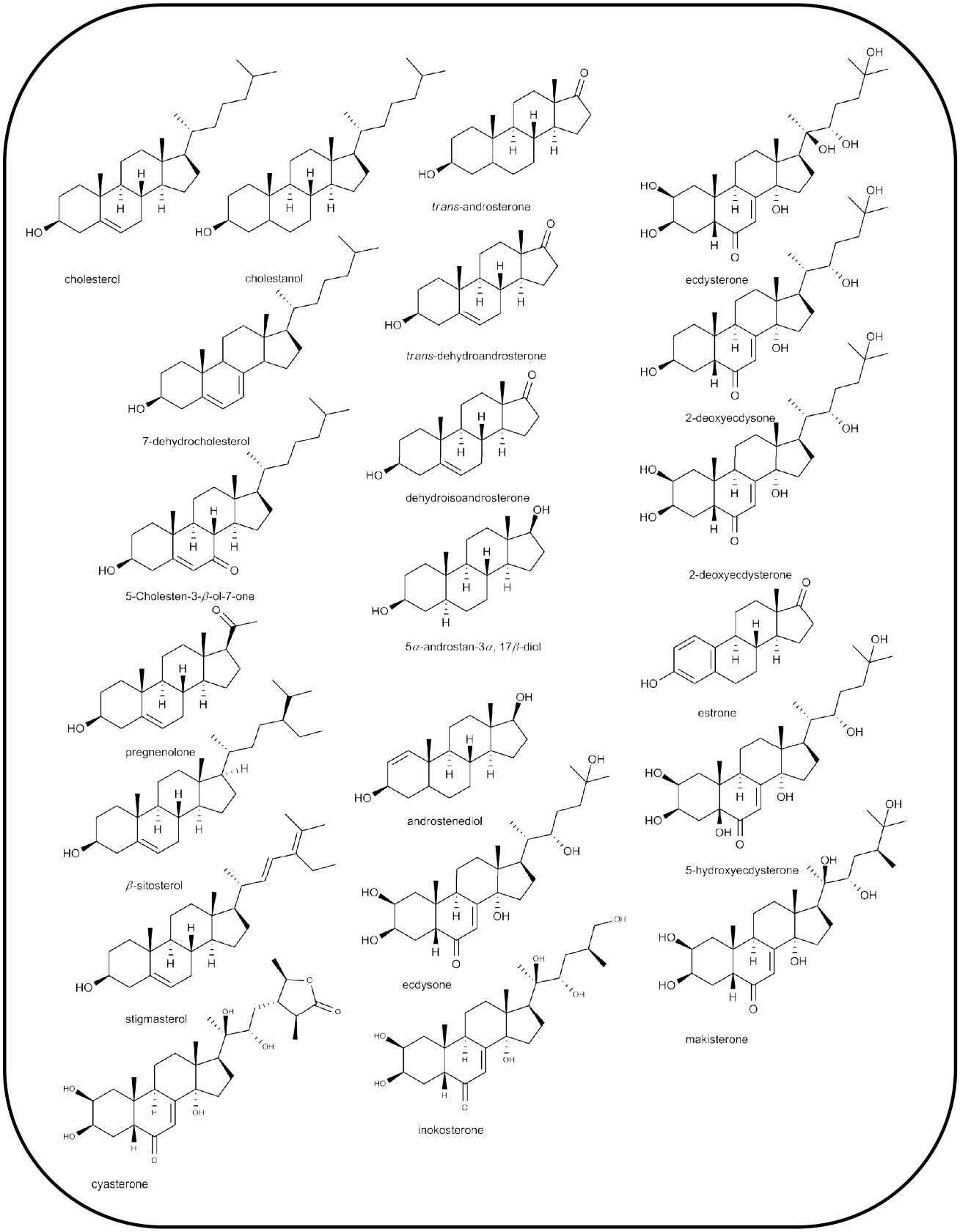



| Substate | Oxidase | Reference |
|---|---|---|
| adenosine | **Nucleoside oxidase[b]** from *F. meningosepticum* | |
| inosine | | |
| guanosine | | [57] |
| thymidine | | |
| uridine | | |
| cytidine | | |
| 2'-deoxyadenosine | **Nucleoside oxidase[b]** from *F. meningosepticum* | [57] |

| | | |
|---|---|---|
| 2'-deoxycytidine<br>2'-deoxyguanosine<br>adenine-9-$\beta$-D-arabinofuranoside<br>6-mercaptopurine arabinoside<br>hypoxanthine-9-$\beta$-D-arabinofuranoside<br>guanine-9-$\beta$-D-arabinofuranoside | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| Tubercidine | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| Kinetine riboside | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| Formycin B | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| Ribavirin | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 8-bromoadenosine | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| adenosine-*N*'-oxide | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 6-($\gamma$, $\gamma$-dimethylallylamino)purine riboside | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 6-benzylaminopurino riboside | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| N-benzoyl-2'-deoxyadenosine | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 2'-tosyladenosine | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 2'-deoxy-1-methyladenosine | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 6-mercaptopurine riboside | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |
| 2, $N^6$-ethenoadenosine | **Nucleoside oxidase**[b] from *F. meningosepticum* | [57] |

[a] Copper-dependent; [b] flavin-dependent

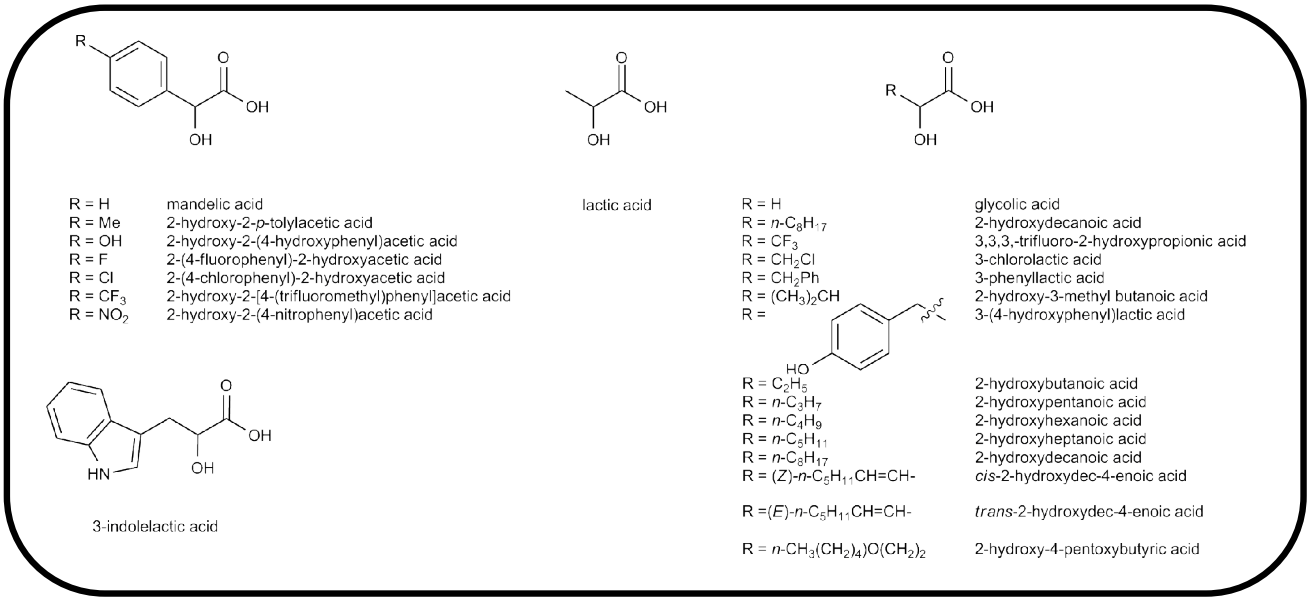
## 2.8. Steroides



| Substrate | Oxidase | Reference |
|---|---|---|
| cholesterol | **Cholesterol oxidase**[b] from *Streptomyces* sp., *B. sterolicum* and *Rhodococcus* sp. | [30] [60] [61] [62] [91] [92] [93] |
| cholestanol | **Cholesterol oxidase**[b] from *B. sterolicum*, *Streptomyces* sp., *Rhodococcus* sp., | [61] [91] [92] |
| 7-dehydrocholesterol | **Cholesterol oxidase**[b] from *Rhodococcus* sp. | [61] |
| 4,5-cyclopropanocholestan-3$\alpha$-ol | **Cholesterol oxidase**[b] from *Streptomyces* sp. | [92] |
| pregnenolone | **Cholesterol oxidase**[b] from *B. serolicum* and *Streptomyces* sp. | [62] [91] [92] |
| $\beta$-sitosterol | **Cholesterol oxidase**[b] from *B. sterolicum*, *Rhodococcus* sp. and *Streptomyces* sp. | [59] [61] |
| stigmasterol | **Cholesterol oxidase**[b] from *B. sterolicum*, *Rhodococcus* sp. and *Streptomyces* sp. | [59] [61] |
| *trans*-androsterone | **Cholesterol oxidase**[b] from *B. sterolicum*, and *S. hygroscopicus* | [91] |
| *trans*-dehydroandrosterone | **Cholesterol oxidase**[b] from *B. sterolicum*, *Rhodococcus* sp. and *Streptomyces* sp. | [59] [61] [91] |
| dehydroisoandrosterone | **Cholesterol oxidase**[b] from *Streptomyces* sp. | [62] |
| 5α-androstan-3$\alpha$,17$\beta$-diol | **Cholesterol oxidase**[b] from *Rhodococcus* sp. | [61] |
| androstenediol | **Cholesterol oxidase**[b] from *R. erythropolis* | [60] |
| ecdysone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| ecdysterone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| 2-deoxyecdysone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| 2-deoxyecdysterone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| 5-hydroxyecdysterone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| makisterone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| inokosterone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |
| cyasterone | **Ecdysone oxidase**[b] from *C. vicina* | [58] |

[a] Copper-dependent; [b] flavin-dependent

## 2.9. α-Hydroxy acids




| Substrates | Oxidases | Reference |
|---|---|---|
| glycolic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [64] [65] [94] |
| lactic acid | **Glycolate oxidase**[b] from *Sp. oleracea;* **Lactate oxidase**[b] from *P. stutzeri* | [64] [65] [67] [94] |
| mandelic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [64] [94] |
| 2-hydroxy-2-(4-hydroxyphenyl)acetic acid | **Lactate oxidase**[b] from *A. viridans* | [64] |
| 2-hydroxy-2-(*p*-tolyl)acetic acid | **Lactate oxidase**[b] from *A. viridans* | [66] |
| 2-(4-fluorophenyl)-2-hydroxyacetic acid<br>2-(4-chlorophenyl)-2-hydroxyacetic acid | **Lactate oxidase**[b] from *A. viridans* | [66] |
| 2-hydroxy-2-[4-(trifluoromethyl)phenyl]acetic acid<br>2-hydroxy-2-(4-nitrophenyl)acetic acid | **Lactate oxidase**[b] from *A. viridans* | [66] |
| 2-hydroxy-2-phenylacetic acid<br>2-methoxy-2-phenylacetic acid | **Glycolate oxidase**[b] from *Sp. oleracea;* **Lactate oxidase**[b] from *A. viridans* | [65] [66] [94] |
| 3-phenyllactic acid<br>3-chlorolactic acid<br>3-indolelactic acid<br>3-(4-hydroxyphenyl)lactic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [65] |
| 2-hydroxy butanoic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [65] [94] |

| | | |
|---|---|---|
| 2-hydroxy-3-methyl hexanoic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [62] |
| 2-hydroxy-4-pentoxybutyric acid<br>2-hydroxyheptanoic acid<br>2-hydroxydecanoic acid<br>*trans*-2-hydroxydec-4-enoic acid<br>*cis*-2-hydroxydec-4-enoic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [65] |
| 3,3,3,-trifluoro-2-hydroxypropionic acid | **Glycolate oxidase**[b] from *Sp. oleracea* | [65] |

[a] Copper-dependent; [b] flavin-dependent

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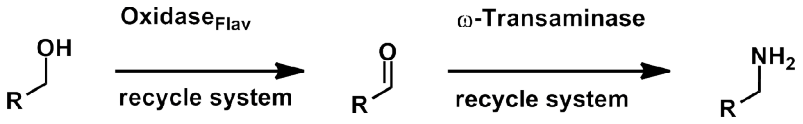
# 3. Research objective

## 3.1. Aim of the thesis

The research object of this thesis was to develop a biocatalytic system in which an alcohol is finally transformed into an amine functionality. For that purpose, a cascade reaction employing flavoprotein oxidases and ω-transaminases was investigated. The flavoprotein oxidase was responsible to convert the alcohol into an aldehyde which was subsequently subjected to reductive amination by the ω-TA, ultimately forming the desired amine (Scheme 13).




**Scheme 13: Oxidation and subsequent amination of an alcohol function**

With the established cascade in hand, optimisation of the protocol concerning reaction time, conversions and reaction conditions was desired. Furthermore, the limited substrate spectrum for the multi-enzymatic system should be investigated.

## 3.2. Definition of the enzymes and establishment of the cascade

A large selection of flavoprotein oxidases and ω-transaminases was available. Most of the enzymes were available in varying expression vectors (Table 1).

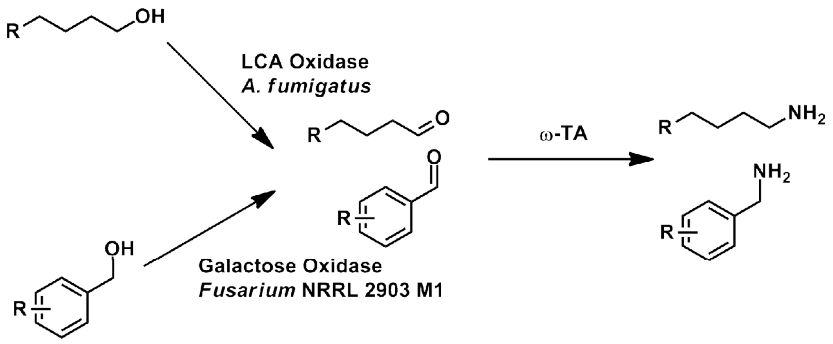
**Table 1: Flavoprotein oxidases and their working plasmid**

| Enzyme | Origin | Working plasmid |
|---|---|---|
| pyranose oxidase | *Trametes ochracea* | pET21a(+) |
| pyranose oxidase | *Phanerochaete chryosporium* | pET16b(+) |
| alditol oxidase | *Chrondus crispus* | pET21a(+) |
| gulonolactone oxidase | *Lysinibacillus spaericus* | pET21a(+) |
| long chain alcohol oxidase | *Aspergillus fumigatus* | pET21a(+) |
| ω-transaminase | *Vibrio fluvialis* | pASK-IBA35(+) |

| | | |
|---|---|---|
| ω-transaminase | *Chromobacterium violaceum* | pET21a(+) |

For protein expression a standard *E. coli* BL2 (DE3) host was transformed with the plasmids and IPTG and ATC were used to induce overexpression. Activity tests were performed using whole cells containing the corresponding plasmid. Previous unpublished studies had shown that the most promising oxidases are pyranose oxidase from *Trametes ochracea* and the long chain alcohol oxidase (LCAO) from *Aspergillus fumigatus*.

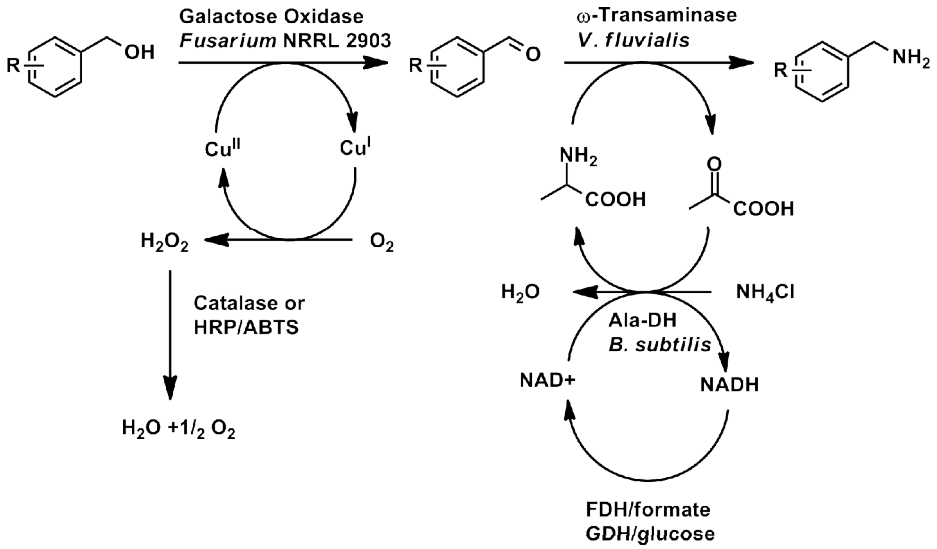
Initially, benzylic alcohols were biotransformed using whole cell preparations of pyranose oxidase, since Fuchs *et al.* already accomplished a cascade system for the latter substrates to obtain the corresponding amine compounds using of copper dependent galactose oxidase from *Fusarium* NRLL 2903 and the ω-transaminase from *Vibrio fluvialis*.[1]. In case that benzylic alcohols would be suitable substrates for pyranose oxidase, the substrate scope might be similar than that of galactose oxidase. In order to get excess to aliphatic alcohols as substrates, long chain alcohol oxidase represented a highly interesting candidate which in a cascade with ω-TA would open the gates to a complementary substrate spectrum to the already established system (scheme 14).




**Scheme 14: Complementary substrate spectrum in a one-pot amination**

As already known from literature, long chain alcohol oxidase showed perfect chemoselectivity towards the oxidation of primary aliphatic alcohols with a certain chain length.[2] Since the enzyme has the ability to over-oxidise the alcohol to the corresponding carboxylic acid, two necessities needed to be given, that the cascade is able to proceed: First, the reductive amination by the ω-TA needed to be faster than the over-oxidation of the aldehyde. Secondly, the aldehyde needed to be accessible for the ω-TA in solution to carry out the reaction.

For the first biotransformation done by this one pot system, already described reaction parameters were used, only the copper (II) was changed to flavin adenine dinucleotide (FAD, Scheme 15) in this system.[1]




**Scheme 15: Amination of benzylic alcohols *via* galactose oxidase and ɤ -transaminase[1]**

In these established conditions a HRP/ABTS assay was employed to remove the cell toxic hydrogen peroxide which was produced as a by-product by oxidases. The main advantage of the HRP/ABTS assay in comparison to catalase induced is a colour change that is due to the homolytic formation of radicals after oxidation.[3] In the system, where FAD was used as a cofactor, the assay was not applicable due to the strong colour of FAD itself. Alternatively, catalase might be used for the cleavage of $H_2O_2$. As standard cascade screening substrate 1-hexanol was chosen as its product (1-hexylamine) was easy to detect after derivatisation. So far, 1-hexanol was not supposed to be oxidised by long chain alcohol oxidases.[2] [4] Initially, the ω-transaminase from *V. fluvialis* was chosen, since the reaction conditions were already optimised for the latter. Furthermore, ω-transaminase from *Chromobacterium violaceum* and from *Paracoccus denitrificans* were both established enzyme candidates for the employment in a multienzyme system. Therefore, a utilisation of these enzymes could improve the process.[1] [5]

## 3.3. Optimisation of reaction conditions

Once there was evidence that the cascade system was working, it was fundamental to further improve the system to achieve optimal conversions. Given that the protein expression was done

in whole cells, it had to be clarified that the oxidation reaction was not partially executed by the alcohol dehydrogenases which were present in *E. coli* expression host. To verify the oxidation potential of the flavoprotein oxidases, their activity needed to be compared to *E. coli* BL21 (DE3) "empty host" not carrying the corresponding oxidase gene sequence.

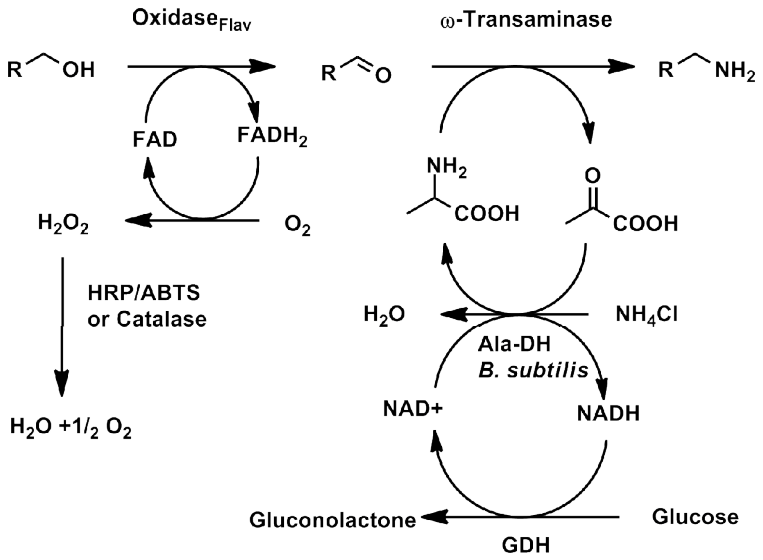
Long chain alcohol oxidase is an oxygen dependent enzyme. Therefore it was important to investigate whether the enzymes were affected by external oxygen sources or whether atmospheric conditions were sufficient enough to carry out the reaction.[2] Another aspect that had to be taken into account, in case oxygen atmosphere was provided, was, whether the solution was saturated with oxygen at all or if a higher volume/surface ratio would improve the saturation of the solution and consequently the conversion of the alcohol moiety.

Another parameter which needed to be investigated for the optimisation of the cascade system was the selection of the biocatalysts (ω-TA and oxidase). Besides the established ω-TA from *V. fluvialis* also the ω-transaminase from *C. violaceum* might be a suitable candidate.[6] The ω-transaminase from *C. violaceum* might be suitable due to its similar behaviour in high pH ranges as expected for the oxidase candidate. pH Studies for the approval of this assumption were required.[7]

Initially both enzymes were used as lyophilised whole cell preparations, however, using cell free extracts of these preparations might have advantages in handling.

As substrate/coenzyme inhibition was often a limiting factor for the activity of certain enzymes, various concentrations of substrates as well as coenzyme (in our case FAD) needed to be tested.

After optimising the conditions to maximise the overall outcome of the biotransformation, time studies would be performed in order to monitor the course of the reaction. Based on those findings the substrate scope of the cascade was investigated.




**Scheme 16: Amination of aliphatic alcohols *via* flavin oxidases and ɤ -transaminases**

## 3.4. Sequence alignment

Much is not known about the long chain alcohol oxidase originating from *Aspergillus fumigatus*.[8] The performance of a sequence alignment would be an approach to substantiate the expected similarities of the described long chain alcohol oxidase from *Aspergillus fumigatus* and the enzyme originating from *Aspergillus terreus*.

# 4. Results and Discussion

## 4.1. Transformation of *E. coli* BL21 DE(3) cells with the corresponding plasmids

The transformation of *E. coli* BL21 (DE3) with the plasmid pET21a(+) containing the gene sequence of the long chain alcohol oxidase from *A. fumigatus* and ω-transaminase from *V. fluvialis* and *C. violaceum*, respectively, was performed successfully.

## 4.2. Overexpression of flavin dependent oxidases and ω-transaminases in *E. coli* and first activity tests

The heterologous overexpression of the oxidases pyranose oxidase from *Phanerochaete chrysosporium*, gulonolactone oxidase from *Lysobacillus spaericus*, long chain alcohol oxidase from *Aspergillus fumigatus*, alditol oxidase from *Streptomyces coelicolor* and hexose oxidase from *Chrondus crispus* and glycerol-3-phosphate oxidase from *Rhodococcus jostii* as well as ω-transaminases from *V. fluvialis* and *C. violaceum* in *E. coli* BL21 (DE3) were performed successfully. To clarify whether the overexpressed enzymes were active, a first activity screening for both enzyme families was performed using already established substrates (Table 2).

**Table 2: First activity screening monitored by a colourimetric assay and GC-MS analysis**

| Enzyme | Substrate | Assay/GC-MS |
|---|---|---|
| alditol oxidase *S. coelicolor*[a] | D-mannitol | - |
| hexose oxidase from *C. crispus*[a] | D-glucose | - |
| pyranose oxidase from *T. ochracea*[a] | D-glucose | ✓ |
| pyranose oxidase from *P. chrysosporium*[a] | D-glucose | ✓ |
| Gulonolactone oxidase from *L. spaericus*[a] | gluconolactone | - |
| Long chain alcohol oxidase from *A.* | heptanol | ✓ |

| | | |
|---|---|---|
| *fumigatus*[a] | | |
| ω-transaminase from *V. fluvialis*[b] | hexanal/benzaldehyde | ✓/✓ |
| ω-transaminase from *C. violaceum*[b] | hexanal/benzaldehyde | ✓/✓ |

[a] Activity was confirmed by colour change using ABTS/HRP assay conditions. Phosphate buffer (100 mM, pH 7.0), oxidase (20 mg whole lyophilised cells), FAD (1 mg/mL), substrate (33 mM), HRP (15 µL), ABTS (15 µL), 16 h at rt, 170 rpm and 1 bar $O_2$; [b] activity confirmed with GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at 30 °C, 120 rpm for 24 h.

In case of the oxidases, activity was determined for pyranose oxidase from *T. ochracea* and *P. cryosporium* as well as for the long chain alcohol oxidase from *A. fumigatus*. In case of the ω-transaminases (*V. fluvialis* and *C. violaceum*) both enzyme candidates showed product formation for both substrates.

## 4.3. Initial conditions and optimised conditions

Several studies were performed to optimise the conditions for the above mentioned standard substates to increase the overall conversion. The initial conditions were taken from previous cascade studies with galactose oxidase from *Fusarium* NRRL 2903 and ω-TA from *V. fluvialis*.[1] Instead of Cu(II) flavin adenine dinucleotide (FAD) was employed as coenzyme in the reaction mixture. For the recycling of $NAD^+$ which was required in the ω-TA path of the cascade, the glucose dehydrogenase (GDH) recycle system was favoured over the formate dehydrogenase system in order to to avoid potential inhibition of the LCA oxidase by formate.[1]

## 4.4. HRP/ABTS assay and catalase

HRP/ABTS and catalase, both ways to degradate the formed $H_2O_2$ in the oxidation pathway, were compared in order to prove their influence on the outcome of the cascade system. The application of the HRP/ABTS assay for the LCA Oxidase/ω-TA system was not reliable, since the detection of the colour change was difficult due to the presence of the strong yellowish flavin cofactor in the solution (Table 3). The applied catalase originates from *Micrococcus lysodeiktikus* (17000 U/mL).

**Table 3: Different methods for hydrogen peroxide cleavage**

| $H_2O_2$ cleavage system | Conversion [%] |
|---|---|
| HRP/ABTS 15 µL | 47 |
| Catalase 10 µL | 47 |

Conversion determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (20 mg whole lyophilised cells), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

The results showed that there was no significant difference of conversion between the application of the catalase from *M. lysodeiktikus* and the HRP/ABTS assay.

## 4.5. Lysis of ω-transaminase and long chain alcohol oxidase

*E. coli* cells containing the overexpressed ω-transaminase from *C. violaceum* or *V. fluvialis* were lysed with lysozyme and subsequently lyophilised. The cell free extract was slightly less active than the whole cell preparation.

After lysis with lysozyme, the *E. coli* cells containing overexpressed long chain alcohol oxidase did not show activity in the cell free extract or in the disrupted cells (Table 4). So a lysis by ultra-sonication was performed and subsequently the cell free extract and the disrupted cells were employed in a biotransformation using initial conditions.

**Table 4: Applying disrupted cells to the cascade**

| Sample | Conversion [$\%_{norm.}$] |
|---|---|
| Cell free extract | 42 |
| Disrupted cells | 26 |

Conversion determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), LCAO (250 µl), FAD (1 mg/mL), ω-TA (10 mg whole lyophilised cells), HRP/ABTS (15 µL), GDH (10 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (5 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

However, both supernatants (ω-TA and LCAO) showed less activity and rather low stability. Therefore, the cascade was performed with whole lyophilised cells.

The whole cell preparation of LCAO from *A. fumigatus* turned out to degrade over time even when stored at 4 °C. So for the substrate screening it was necessary use rather freshly prepared whole lyophilised cells. Therefore, the obtained conversions for the optimisation studies were normalised. The best conversion obtained was set to 100% and all further measurements were calculated relatively to it.

## 4.6. Over-oxidation of aliphatic alcohol with long chain alcohol oxidase

FAD dependent oxidases did not necessarily stop the oxidation of the substrate at the formation of an aldehyde, instead they were able to further oxidise the substrate to the corresponding carboxylic acids. This over-oxidation process was well investigated for several flavin dependent oxidases.[9] In order to gain more insight regarding the over-oxidation of the corresponding aldehyde, experiments with and without additional FAD were performed and substrate 1-hexanol was applied as substrate (Table 5).

**Table 5: Over-oxidation of the substrate using long chain alcohol oxidase with or without additional FAD**

| Sample | 1-Hexanol [%] | Hexanal [%] | Hexanoic acid [%] |
|---|---|---|---|
| LCAO with FAD | 64 | 26 | 10 |
| LCAO without FAD | 84 | 16 | Traces |
| „empty host“ with FAD | >99 | traces | - |
| „empty host” without FAD | >99 | traces | - |

Determined by GC-MS analysis. Phosphate buffer (100 mM, pH 7.0), whole lyophilised cells (20 mg), FAD (1 mg/mL), catalase (10 µL) 20 h at rt, 170 rpm and 4 bar $O_2$.

The over-oxidation to the corresponding acid was detected when FAD was added and was only negligible without addition of the cofactor. This indicates that for the reductive amination, the ω-transaminase intercepted the aldehyde intermediate obtained from the oxidation process to form the amine. This observation led to the assumption that after leaving the active site, the aldehyde acted as a substrate for both, the ω-transaminase and the LCAO for the acid formation. Consequently, the ω-transaminase pulled the equilibrium towards product formation.

In case of the long chain alcohol oxidase a significant increase in the conversion was observed when additional FAD was employed. This might be due to several reasons. Either the enzyme uses the supplemented FAD in addition or more likely the enzyme compensates the loss of FAD. Control studies showed that free FAD is not able to carry out the oxidation of the aliphatic alcohols.

Additionally, studies were performed containing the whole recycling system for the ω-TA to determine the influence on substrate stability and conversion. However, in these tests no formation of aldehyde or carboxylic acid could be observed, so it was likely that the recycling system somehow influenced the oxidation negatively.

## 4.7. FAD concentration study

The availability of coenzymes can act as a limiting factor for many biotransformations in terms of the conversion. So it was intriguing whether the conversion was increased with additional FAD in the multienzyme system or whether inhibition took place. Concentrations from 1 to 4 mg/mL as well as no FAD addition to the buffer solution were tested. The biotransformations were performed under initial screening conditions (pH 7.0, 20 h, 4 bar $O_2$) using ω-TA from *C. violaceum* instead of ω-TA from *V. fluvialis* (Table 6).

**Table 6: Variation of FAD concentration in the cascade system**

| FAD Concentration [mg/mL] | Conversion [%norm.] |
|---|---|
| 0 | <99 |
| 1 | 70 |
| 2 | 51 |
| 4 | 54 |

Conversions determined by GC-MS analysis after derivatisation and normalised. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), LCAO (20 mg whole lyophilised cells), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

A decrease in conversion was observed when the FAD concentration was increased. Surprisingly, the best results were obtained without any additional FAD. It seems that long chain alcohol oxidase carrying already inherently a flavin, either covalently bound or fixed by the tertiary structure of the enzyme. Even though this is contradictory, for further biotransformations FAD was still added. Based on those results the question rose whether FAD in solution inhibited the ω-transaminase or an enzyme of the recycling system. In order to gain more insight further studies are required.

## 4.8. ω-Transaminase

Besides the already established ω-transaminases, the ω-transaminase from *Paracoccus denitrificans* was introduced in the cascade system with LCAO. The initial screening conditions were applied using 1-hexanol as test-substrate (Table 7).

**Table 7: Amination of 1-hexanol using LCAO in combination with various ω -TAs**

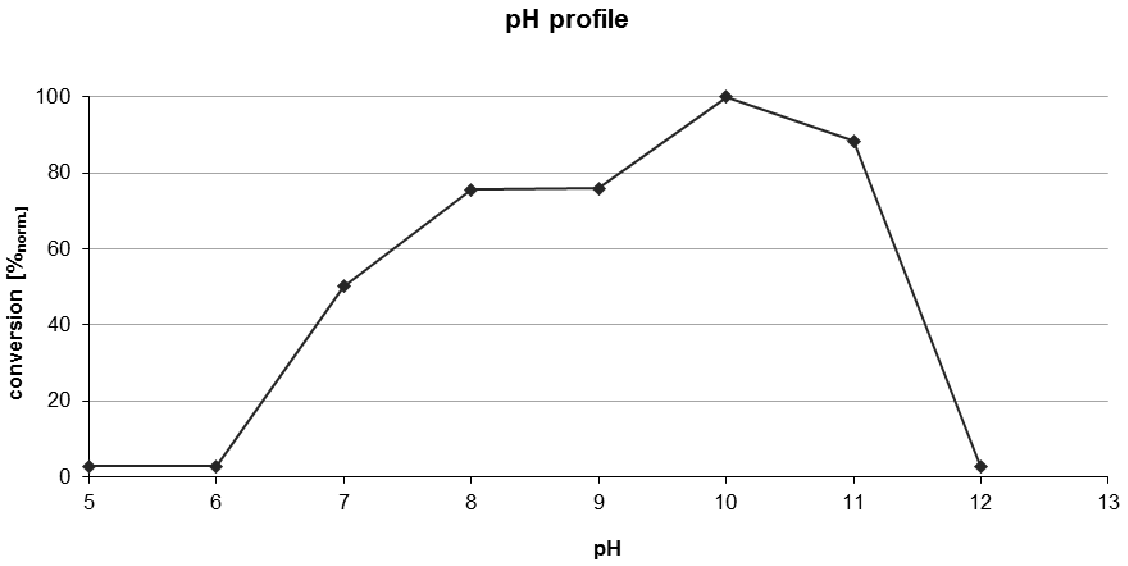
| ω-Transaminase | Conversion [%] |
|---|---|
| *V. fluvialis* | 49 |
| *C. violaceum* | 48 |
| *P. denitrificans* | 20 |

Conversion determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), LCAO (20 mg whole lyophilised cells), FAD (1 mg/mL) ω-TA (20 mg whole lyophilised cells), HRP/ABTS (15 µL), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

The ω-transaminases from *V. fluvialis* and *C. violaceum* converted the substrate without significant difference (conv. = 49% and 48%, respectively). The newly introduced ω-TA from *P. denitrificans* led to a significant drop in conversion (conv. = 20%) and was not further investigated.

## 4.9. pH study

It was reported in literature that the pH value of the reaction solution might play an important role in the activity of an alcohol oxidase as well as for the ω-TA from *C. violaceum*.[7] [10] A pH study was therefore performed to optimise the screening conditions and to get an idea about the pH profile of the system. For that reason phosphate buffer was adjusted to the desired pH values [pH 4.0 - 12.0 in full pH steps (data shown in Figure 1)]. The initial screening conditions and the standard test substrate 1-hexanolwere applied.



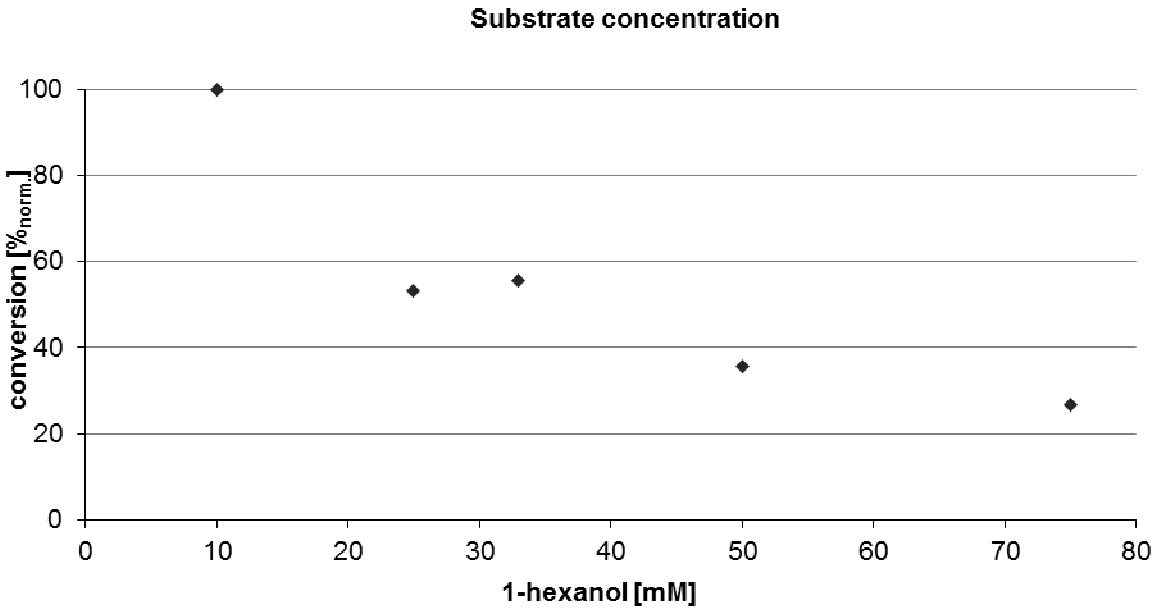

Conversions determined by GC-MS analysis after derivatisation and normalised. Phosphate buffer (100 mM), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (20 mg whole lyophilised cells), ɤ -TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

**Figure 1: pH profile of the amination cascade employing LCAO and ɤ ï TA**

The cascade system was most efficient at basic conditions at 10.0 pH with >99% normalised conversion of 1-hexanol to the corresponding amine. Compared to the initially introduced conditions at pH 7.0 where only 50% conversion was obtained, the new pH adjustments exhibited a significant increase in conversion. This might seem surprising, but the ω-TA from *C. violaceum* and the LCAO from *A. terreus,* showed both a strong preference for a basic environment.[7] [11]

## 4.10. Substrate loading

Since the substrate concentration is of a crucial interest in a biocatalytic application, various concentrations of the alcohol substrate were applied in the cascade system. A loading of 10 mM substrate was defined as lower limit and the loading was increased stepwise up to 75 mM. The screening was performed under initial conditions with the standard test substrate (data shown in Figure 2).




Conversions determined by GC-MS analysis after derivatisation and normalised. Phosphate buffer (100 mM), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (20 mg whole lyophilised cells), ɤ -TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol),), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

**Figure 2: Variation of the substrate loading (10 mM, 25 mM, 33 mM, 50 mM, 75 mM)**

The best conversion was obtained employing 10 mM substrate loading. To improve the conversions of our system the same substrate loading was used in the optimised conditions.

## 4.11. Ratio of ω-transaminase and long chain alcohol oxidase in the cascade system

It was assumed that the long chain alcohol oxidase acted as the limiting factor in the multi-enzyme process. Different ratios of LCAO/ω-TA were tested (1:1 as reference, 2:1, 2.5:1, 3:1). (Table 8). Initial screening conditions and the standard test substrate 1-hexanol were applied.

**Table 8: Ratios of long chain alcohol oxidase and ɤ ï transaminase**

| Ratio LCAO/ω-TA | LCAO [mg] | ω-TA [mg] | Conversion [%norm.] |
|---|---|---|---|
| 1:1 | 20 | 20 | 60 |
| 2:1 | 40 | 20 | 67 |

| | | | |
|---|---|---|---|
| 2.5:1 | 50 | 20 | 87 |
| 3:1 | 60 | 20 | <99 |

Conversions determined by GC-MS analysis after derivatisation and normalised. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

The data indicated that additional oxidase indeed improved the conversion of the substrate to the corresponding amine. The conversion was increased by 40 % when the amount of LCAO was increased threefold.

## 4.12. Application of different oxygen pressure levels onto the multienzyme system

The supply of the co-substrate oxygen was expected to be a limiting factor enzymatic system. Furthermore, it was of interest to see whether atmospheric conditions were sufficient enough or if oxygen pressure (requiring an apparatus) was needed. Various pressures of oxygen as well as atmospheric conditions were applied to the cascade system using initial screening conditions and the standard test substrate (Table 9).

**Table 9: Different oxygen supply for cascade system**

| Pressure | Conversion [$\%_{norm.}$] |
|---|---|
| Atm (bench) | 40 |
| 1 bar $O_2$ (apparatus) | 88 |
| 2 bar $O_2$ (apparatus) | <99 |
| 3 bar $O_2$ (apparatus) | 96 |
| 4 bar $O_2$ (apparatus) | <99 |
| 5 bar $O_2$ (apparatus) | 60 |

Conversions determined by GC-MS analysis after derivatisation and normalised. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (20 mg lyophilised w.c.), ω-TA (20 mg lyophilised w.c.), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt and 170 rpm.

A significant increase of conversion was obtained by putting the reaction mixture under pure oxygen atmosphere. Up to two bar an improvement of conversion was observed, whereas similar levels of conversions were obtained with further increasing of the $O_2$ pressure. The decrease when the highest pressure was applied was expected due to cell damage.

## 4.13. Influence of surface size

Although a saturation of the system with oxygen was indicated when using standard conditions, we wanted to investigate whether the size of the surface had any influence. Therefore, two different vessels were used: A 10 mL flask and a MG5 vial (4 mL).

Both samples showed the same conversion values meaning that the influence of size of surface was negligible. This experiment proved the assumption that the applied pressure was sufficient enough to saturate the solution with oxygen.

## 4.14. Control reaction with empty whole cells

In the *E. coli* host alcohol dehydrogenases (ADHs) were inherently present at a relevant level.[11] These enzymes had a broad substrate acceptance which might lead to a significant background reaction. To clarify whether the oxidation of substrate was performed by the overexpressed enzymes or by the *E. coli* host, control reactions with empty host were performed. Also in case of background reaction the fast reductive amination of the ω-transaminase seemed to be the driving force. Therefore the conversions in the control reaction were significantly higher, compared to the one step oxidation reaction.

ADHs were not just known to oxidise alcohols, but also to reduce carbonyl compounds it was interesting, whether they were also able to reduce the aldehyde functionalities. Therefore, whole *E. coli* cells were employed using eighter 1-hexanol or the corresponding aldehyde 1-hexanal as substrates.[12]

**Table 10: Applying empty E. coli BL21 (DE3) host for background reaction**

| Substrate | Alcohol [%] | Aldehyde [%] |
|---|---|---|
| 1-hexanal | traces | n.q. |
| 1-hexanol | >99 | traces |

Determined by GC-MS analysis. Phosphate buffer (100 mM, pH 7.0), *E. coli* BL21 (DE3) (40 mg, whole lyophilised cells), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), 20 h at rt, 170 rpm and 4 bar $O_2$.

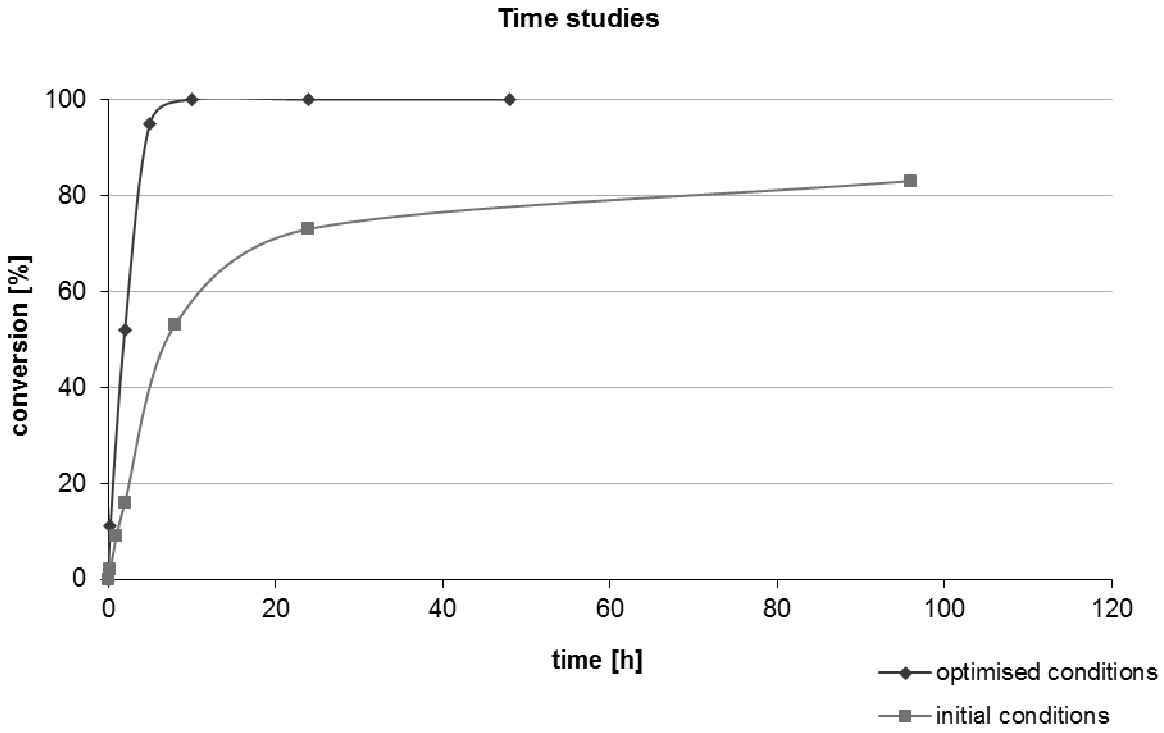
As displayed in Table 10, ADHs were able to act as oxidants as well as reductants. Consequently they were able to push the reaction 'forward', but if the equilibrium is on the aldehyde side, a competing 'backward' reduction occured.

## 4.15. Denaturation experiment

In order to verify that the observed oxidation/amination activity was derived from the biocatalysts various denaturation experiments were performed under optimised screening conditions. The samples were prepared identical to the biotransformation (optimal screening conditions) and treated with heat (80 °C for 20 min) or SDS (1% added after rehydration). Furthermore, samples without cells (LCAO and ω–TA) or substrate were tested for product formation. No product formation was detected in any case.

## 4.16. Time studies

Time studies were performed using either the initial screening conditions of the biotransformation or the optimised screening conditions which led to a significant improvement of the biocatalytic outcome. Whereas in the experiment using the initial condition a strong loss of activity after 24 h was observed and complete conversion was never achieved (Figure 3), the reaction with the optimised conditions lead to full conversion after already 10 h.




Determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 7.0 or pH 10.0, respectively), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (20 mg whole lyophilised cells or 40 mg, respectively), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM or 10 mM, respectively), Ala-DH (10 µL), at rt, 170 rpm and 4 bar or 2 bar $O_2$, respectively.

**Figure 3: Comparision of the time studies using initial and optimised screening conditions**

## 4.17. Substrate screening for LCAO/ω-TA cascade system

### 4.17.1. Aliphatic alcohols

For LCA oxidase different fatty alcohols were reported as natural substrates.[4] [6] Since the substrate scope is of crucial importance for a biocatalytic application, the substrate tolerance of the cascade system was investigated. 1-Hexanol was chosen as standard substrate to establish and optimise screening conditions. Four other alcohols with different chain lengths were tested as substrates for the cascade under optimised conditions (Table 11).

**Table 11: Substrate scope of the cascade system employing saturated, unsubstituted aliphatic alcohols**

| Substrate | Conversion [%] |
|---|---|
| 1-pentanol | 75 |

| | |
|---|---|
| 1-hexanol | >99 |
| 1-heptanol | >99 |
| 1-octanol | >99 |
| 1-nonanol | 82 |

Determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 10.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (40 mg whole lyophilised cells), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (10 mM), Ala-DH (10 µL), for 24 h at rt, 170 rpm and 2 bar $O_2$.

All applied aliphatic alcohols were suitable substrates for the cascade system. Full conversions under optimised conditions were obtained for 1-hexanol, 1-heptanol and 1-octanol to furnish the corresponding amines. 1-Pentanol, which was not described as canonical substrate for long chain alcohol oxidase, was fortunately, accepted by the enzyme (conv. = 75%). Furthermore, 1-nonanol, the substrate with the longest tested hydrocarbon backbone was also accepted as a substrate (conv. = 82 %). For the latter traces of the corresponding acid (nonanoic acid) were detected. So it was likely that the competing oxidative formation of the carboxylic acid limited the conversion. The results indicated that the chain length of the substrate had a significant influence on the substrate tolerance of LCAO. Longer hydrocarbon backbones seemed to over-oxidise faster than shorter ones.

### 4.17.2. ω-Halogenated alcohols

The ω-halogenated substrates, which were applied as substrates, had chain lengths of six to eight carbon atoms, respectively (i.e. 6-chlorohexanol, 8-chlorooctanol, 6-bromohexanol and 8-bromooctanol, Table 12). The biotransformations were performed with optimised screening conditions.

**Table 12: Substrate scope of the cascade employing ω-halogenated aliphatic alcohols**

| Substrate | Conversion [%] |
|---|---|
| 6-chlorohexanol | 98 |
| 8-chlorooctanol | 96 |
| 6-bromohexanol | 74 |
| 8-bromooctanol | n.q. |

Determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 10.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (40 mg whole lyophilised cells), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (10 mM), Ala-DH (10 µL), for 24 h at rt, 170 rpm and 2 bar $O_2$.

In case of those substrates, product formation was detected with GC-MS after derivatisation with ethyl(succimidooxy)formate. 6-Chlorohexanol, 8-chlorooctanol and 6-bromohexanol were accepted showing good to excellent conversions (conv. 74% - 98%) to the corresponding amine products. The conversion for 8-bromooctanol was reasonable, however, due to analytical problems the obtained product was not quantified.

## 4.18. Unsaturated alcohols

Unsaturated alcohols with *E/Z*-configuration are considered as interesting substrates (i.e. *trans*-3-hexenol and *cis*-2-hexenol). The reduced flexibility of the hydrocarbon chain through the double bond was expected to influence the substrate acceptance of the LCAO. Furthermore, it was of interest whether the enzymes would differ between the *trans*- and the more sterically demanding *cis*-configuration of the substrates in terms of conversions.

Unfortunatly, none of the applied unsatured alcohols, neither in *cis*- nor in *trans*-configuration, were accepted as substrate by the system. This led to the conclusion that the flexibility of the chain was essential to fit into the active site of LCAO from *A. fumigatus* since ω-TA from *C. violaceum* was described to aminate also sterically rigid substrates.

## 4.19. Further substrates and limits of the system

Terpene alcohols (such as geraniol), benzyl alcohol and secondary alcohols (such as 2-heptanol) were not expected as substrates for the cascade system (Table 13). The substrates were tested under optimised screening conditions.

**Table 13: Benzyl alcohol, terpene alcohol (geraniol) and secondary alcohol (2-heptanol) applied as substrates in the cascade system**

| Substrate | Conversion [%] |
|---|---|
| benzyl alcohol | traces |
| geraniol | n.c. |
| 2-heptanol | traces |

Determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 10.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (40 mg whole lyophilised cells), ω-TA (20 mg whole lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (10 mM), Ala-DH (10 µL), at 24 h, rt, 170 rpm and 2 bar $O_2$.

In case of benzyl alcohol and 2-heptanol, traces of the corresponding amine could be detected, however, the empty host blank gave the same results, which consequently meant that the conversion was derived from the enzymatic background reaction in *E. coli* host.

With the initial screening conditions several more alcohols were tested for conversion to the corresponding amines without any success (Table 14).

**Table 14: Further alcohol substrates without conversion which showed no conversion**

| Substrates | Conversion [%] |
|---|---|
| 2-octyn-1-ol | n.c. |
| nerol | n.c. |
| 6-aminohexanol | n.c. |
| 8-phenyl-1-octanol | traces |
| *trans*-2-decen-1-ol | n.c. |

| | |
|---|---|
| 2-decyn-1-ol | n.c. |

Determined by GC-MS analysis after derivatisation. Phosphate buffer (100 mM, pH 7.0), L-alanine (0.15 mmol), NADH (2 mM), $NH_4Cl$ (0.1 mmol), PLP (2 mM), FAD (1 mg/mL), LCAO (20 mg whole lyophilised cells), ω-TA (20 mg whle lyophilised cells), GDH (20 µL), glucose (0.12 mmol), substrate (33 mM), Ala-DH (10 µL), at rt, 170 rpm and 2 bar $O_2$.

## 4.20. Application of pyranose oxidase in a cascade system

Besides long chain alcohol oxidase from *A. fumigatus*, also pyranose oxidases from *T. ochracea* and *P. chryosporium* were employed in the oxidation reaction of the multienzymatic cascade system. The enzymes were used as whole lyophilised cell preparation. Benzyl alcohol was used as a test substrate for the biotransformation which were performed under initial conditions (pH 7.0, ω-TA *V. fluvialis*, 20 h, 4 bar $O_2$). For GC-MS analysis the samples were split for detection of the corresponding acid and amine, respectively. The amine was detected after derivatisation using ESOF.

The corresponding amine product was detected in with both pyranose oxidase species. Furthermore, some hints for the presence of benzaldehyde and benzoic acid were found. However, it was not completely clear, if the oxidation was performed by the overexpressed oxidase or the alcohol dehydrogenases present in the host, however, since traces of acid were detected, which was mainly observed for flavin oxidases, the oxidation possibly occured by pyranose oxidase.

## 4.21. Sequence alignment

The sequences of long chain alcohol oxidase from *Aspergillus fumigatus* (accession number XP_753079.1) and from *Aspergillus terreus* (accession number XP_001214264.1) were aligned. The sequences shared a sequence identity of 70 %.

```
AfumigatusLCAO      MAEQAVTAYVPLDVPLPPIPEGQVFSDLQWRTLLSLADTVIPSIRSTSLPKSVSTKVVPE 60
AterreusLCAO        MTDQAV-AYTPLDVALPPVPTTEVFSDLQWTTLLALADTVIPSIAP-SAPKSRAAKVISQ 58
                    *::*** **.****.***:*  :******* ***:********* . * *** ::**:.:

AfumigatusLCAO      STFKDAVSTLASHIHDPDATQIAEQYLEENASANPQFVEGLRRLFAEYIHEEGKSGINLI 120
AterreusLCAO        SEYDAVHSDLVARIHAPNASELATQYLEEHASSNPGFRDGIQRLFANYVHQEGRNGISLI 118
                    * :. . * *.::** *:*:::* *****:**:** * :*::****:*:*:**:.**.**

AfumigatusLCAO      LNALNSKAGSLILTGSTTPIQDQPFEIREKIFSSWETSRIKPLRAIYRAFTAIFKKTWTV 180
AterreusLCAO        LTALNTKAGSLILTGSITPIQDQPLEYREQVFRGWETSRLRPLRAVYRALSGIFKRTWIV 178
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                  *.***:********** *******:* **::* .*****::****:***::.***:** *

AfumigatusLCAO    S-PTIRSVVGCPRVPIHGKPADGFEYEFLQFPPGAEPETIDTDVVIVGSGCGGSVAAK-L 238
AterreusLCAO      SSPTICPVIGFPRVPVHGKPEDGFPYQFLQIPPGDEPETIETDVVIVGSGCGGGVTAKNL 238
                  * *** .*:* ****:**** *** *:***:*** *****:************.*:** *

AfumigatusLCAO    AEAGYRVLVVEKSYHYPSKYFPMDFNEGFVSMFENGGATTSDDGSIAVLAGSTWGGGGTV 298
AterreusLCAO      AEAGHKVLVVEKGYQYSSRHFPMGFNEGLNSMFEASAATGTDDGTMGLFAGSTWGGGGTV 298
                  ****::******.*:*.*::***.****: **** ..** :***::.::*********** 

AfumigatusLCAO    NWSASLQTQGYVRREWASKGLPFFESHEYQQALDRVCDRMGVSNDHTEHNYSNRVLLDGA 358
AterreusLCAO      NWSASLQTQGYVRQEWADAGLPFFTSFEFQRCLDRVCDRMGVSDEHQDHNFQNRMLLEGA 358
                  *************:***. ***** *.*:*:.***********::* :**:.**:**:** 

AfumigatusLCAO    RKLGYAAQPVPQNTGGSNHYCGYCTMGCHSCGKKGPRETFLADAAKAGTTFIEGFRADKI 418
AterreusLCAO      RKLGYAAKPVPQNTGGTGHYCGYCTMGCHSTGKKGPTESWLADAAKAGATFMEGFRADKV 418
                  *******:********:.************ ***** *::********:**:*******: 

AfumigatusLCAO    RFKNTKGGRVACGVEGTWTSRDSYLGTAGPDRTTRKVIINASKVIVSCGTLHSPLLLLRS 478
AterreusLCAO      LFDNTKGGQVASGVEGTWTSRDSYLGLSGEGARKRKVIIKAKKVVVACGSLQSPLLLLRS 478
                   *.*****:**.************** :* .  .*****:*.**:*:**:*:******** 

AfumigatusLCAO    GLKNPQIGRNLYLHPVVLSCAVFDEEIRPWEGSALTIVVNEFEDQDGQGHGVKIENVVML 538
AterreusLCAO      GLKNSQIGRNLYLHPVVLATAVFEEETRPWEGACLTTVVNELEDQDGKGHGVKIECVTML 538
                  ****.*************: ***:** *****:.** ****:*****:******* *.** 

AfumigatusLCAO    PALYLPTFPWRDGLDYKLWAAKLPRMSGFIALTKERDAGRVYPDPADGRVRIDYTVSAYD 598
AterreusLCAO      PPAFLPAFPWRDGLDYKKFVAKLPHMGGFIMLTRDRDSGRVYPDPVDGRCRVDYSVSSYD 598
                  *. :**:********** :.****:*.*** **::**:*******.*** *:**:**:** 

AfumigatusLCAO    RKHIVEALIATAKIAYISGAREFHTSNREMPPFIRPTEASDPNAPEGVTNQALQAWIAVL 658
AterreusLCAO      RNHMVEALAASAKIAYISGAKEFHTSCRGLPPFIRPAEA-DADDAEGTNNAALQSWLAEL 657
                  *:*:**** *:*********:***** * :******:** *.: .**..* ***:*:* * 

AfumigatusLCAO    RRKNPVDPERTQYASAHQMGTCRMGSSPRTSVVDPECQVWGTQGLYVMDASVFPSASGVN 718
AterreusLCAO      RRK-ALEPERTLFACAHQMGSCRMGKSPASSVVDPDCQVWGTKGLYVMDASVFPSASGVN 716
                  *** .::**** :*.*****:****.** :*****:******:***************** 

AfumigatusLCAO    PMVTNMAIADWASRKVVRSLEKANHGKTVLARL 751
AterreusLCAO      PMVTNMAIADFASRGLAKTLSKEKKEGAPVARL 749
                  **********:*** :.::*.* ::  : :***
```
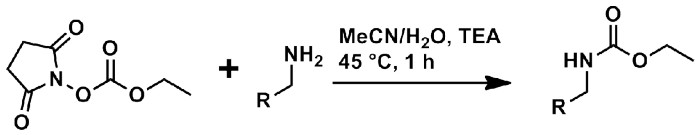

Two large conserved domains were revealed. The biggest conserved domain was the flavin binding domain from amino acid was found at amino acid 272 up to amino acid 496. This domain was found in a similar region in the LCAO from *A. terreus*. The second conserved domain, an oxidoreductases superfamily domain, was found at amino acid 575 up to 730.

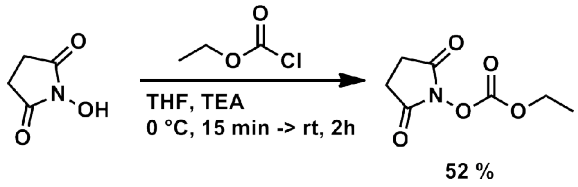
## 4.22. Ethyl(succinimidooxy)formate

Derivatisation of the formed amines was necessary, because the underivatised compounds were not detectable with the applied GC-MS analysis. Therefore, the formation of an ethyl carbamate with ethyl(succinimidooxy)formate (ESOF) as derivatisation reagent was the method of choice (Scheme 17), in which the hydroxysuccinimide worked as leaving group in the derivatisation reaction.




**Scheme 17: Derivatisation of the amine product to the corresponding ethyl carbamate using ESOF as derivatisation reagent**

The synthesis of ethyl(succinimidooxy)formate was performed using a procedure reported by Edafiogho *et al*. (Scheme 18).[13]




**Scheme 18: Synthesis of ethyl(succinimidooxy)formate (ESOF)**

Even though the yield was only moderate after recrystallization (52 %), the NMR spectrum showed no impurities which made it sufficient for the use as derivatisation reagent.

## 4.23. Conclusion

The establishment of a cascade system employing a flavin dependent oxidase and a ω-transaminase was successfully achieved by using the long chain alcohol oxidase from *A. fumigatus* and a ω-transaminase from *C. violaceum*. After conversion of the test substrate 1-hexanol was observed, optimisation studies were performed and the substrate scope was investigated. While the substrate scope was not as broad as expected, good to excellent conversions were obtained for several aliphatic and ω-halogenated alcohols. Furthermore, a sequence alignment was performed to get a better insight in the enzymes structure, since LCAO from *A. fumigatus* was almost undescribed in literature.

# 5. Outlook

Since the cascade system for aliphatic alcohols is now established a further improvement of the single parts of the cascade and its recycling system is desirable.

The process of over-oxidation and consequently the mechanism for long chain alcohol oxidase is not completely understood. Therefore further experiments are desirable to prove the influence of the chain length and how reductive amination is influenced by these conditions.

For further optimisation of FAD concentrations experiments are still required, for example how the total deletion of flavin from the enzyme would influence catalytic behaviour of the LCAO. A main focus should be set on the optimisation, as FAD is a costly cofactor. Furthermore, FAD might inhibit ω-transaminases, therefore, inhibition studies could clearify this assumption. The GDH recycling system turned out to work well for the long chain alcohol oxidase/ω-transaminase system and was used to avoid expected inhibition problems. Still it has the disadvantage of a rather low atomic efficiency. To make the process more sufficient, the employment of other recycle systems could lead to an improvement. However, also the recycling process by itself could be optimised such as loading of the cosubstrates.

Since two different whole cell preparations were used, a simplification of the system is desirable. Therefore, the coexpression of the oxidising long chain alcohol oxidase and the reductively aminating ω-transaminase in one single expression host is an option. For the subcloning in a pETDuet-1 vector and the subsequent transformation and overexpression in an *E. coli* BL21 (DE3) host, the first experiments were already performed. When the host is received, expression engineering might be necessary since the overexpression in a duet-vector system could be insufficient for the needs of a cascade process since it is reported that with petDuet-1 vector systems lower expression levels are observed.[14] With an appropriate biocatalyst an upscale even to biotechnological scales might be considered. Another approach would be the use of a so called 'Gibson assembly' to form a plasmid construct, which could lead to a biocatalyst carrying the two overexpressed enzymes.[15]

The substrate scope of the cascade is limited due to solubility problems of the substrate. The possibilities of using co-solvents are limited due to the formation of dangerous peroxides under oxygen pressure. Therefore, the system is limited to solvents like DMSO, ethyl acetate, chloroform etc. Another approach would be the use of ionic liquids which are considered as "green" and are of great interest as they would amplify the approach of a eco-friendly process.[16]

Another way to overcome the solubility problem is the use of micelles forming non denaturating emulsifiers {such as 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), choline chloride, sodium deoxycholate etc.}.[17] A possible problem is the high ion strength and the strong basic pH under optimised conditions which could decrease the effect of the emulsifiers.

Aliphatic alcohols with a longer hydrocarbon backbone represent interesting substrates, since long chain alcohol oxidase is expected to accept the latter as substrates.[2] Furthermore, substrates which are sterically demanding at the ω-position would indicate, how long the flexible aliphatic chain must be for substrate acceptance of the oxidase. For that reason simple phenyl or branched groups are possible candidates. Industrially relevant examples of such ω-substituted compounds are ω-hydroxy fatty acids which are used as building blocks in polyamide synthesis.

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# 6. Experimental

## 6.1. General

All chemicals were purchased from Sigma Aldrich or Acros Organics and used as received. Solvents were obtained from Roth. Rehydration of enzymes and biocatalytic reactions were performed in a HT Infors Unitron AJ 260 incubator at 120 rpm shaking (horizontal position) and 30 °C or in an oxygen pressure chamber apparatus. Centrifugation was done at 13000 rpm in a Heraeus Biofuge pico or at 4000 rpm in a Heraeus Biofuge primo. Conversions were determined by GC-MS analysis on an Agilent 7890A GC system, equipped with an Agilent 5975C mass selective detector (EI 70 eV) and a HP-5-MS column (30 m x 0.25 mm x 0.25 µm film) using He at a flow rate of 0.5 mL/min. Temperature program: 40 °C for 2 min, 20 °C/min to 180 °C for 1 min, 20 °C/min to 300 °C for 4 min. Run time was 20 min. Inlet temperature was 250 °C. Plasmids of alanine dehydrogenase from *Bacillus subtilis* were kind gifts of Arne Skerra (TU Munich). Activity of the alanine dehydrogenase was tested via the transformation of L-alanine to pyruvate (7.5 U/mL). Experiments under oxygen pressure were conducted in a plexiglass cylinder (27 cm length × 10 cm diameter, see below). Horseradish peroxidase (HRP) and catalase from *Micrococcus lysodeikticus* were purchased from Sigma Aldrich (HRP: # P8125, EC 1.11.1.7; Catalase: # 60634, EC 1.11.1.6) and glucose dehydrogenase (GDH) was obtained from DSM (# GDH 0.001).

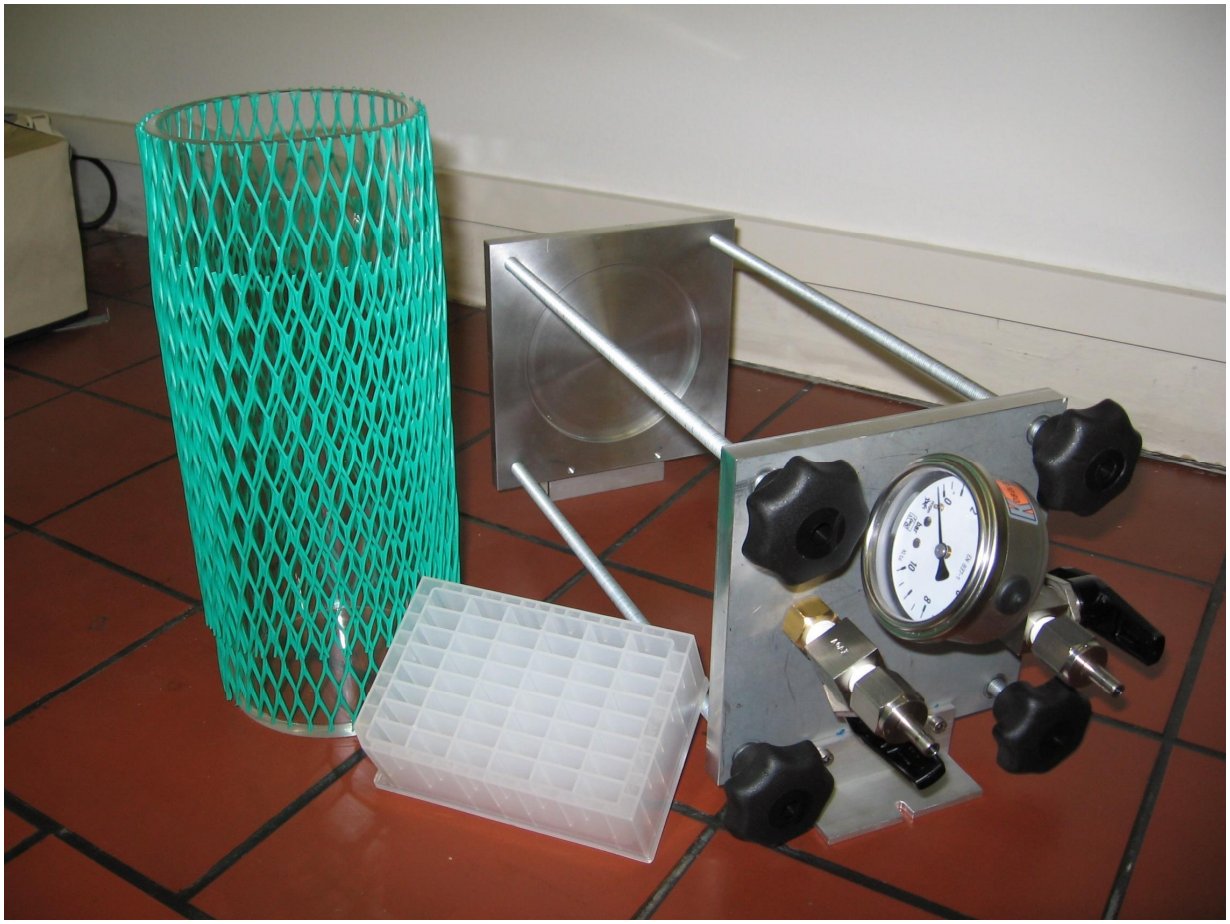

**Figure 4: Oxygen apparatus (plexiglass cylinder 27 cm length × 10 cm diameter)**

## 6.2. Transformation of *E. coli* BL21 (DE3) cells or *E. coli* TOP 10 cells with the corresponding plasmid

Transformation was performed according to the protocol of Quiagen. Plasmid solution (3 µL) was added to chemically competent *E. coli* BL21 (DE3) cells (50 µL) or *E. coli* TOP 10 cells (50 µL) in test tubes. The mixtures were incubated on ice for 30 min and subsequently heated for 42 °C. Then SOC medium (250 µL) was added and the tubes were shaken for 1 h at 37 °C and 120 rpm. After the transformation the mixture was centrifuged with 12000 rpm for 2 min and 200 µL supernatant were discarded. The pellet was resuspended in buffer and streaked on LB/Amp agar plates (1 × 20 µL, 1 × 50 µL, 1 × rest) and incubated overnight at 30 °C.

## 6.3. Preparation of overnight cultures

Freshly prepared LB-medium (10 mL) supplemented with ampicillin (100 µg/mL) was inoculated with a freshly picked one cell colony previously grown on a LB-agar plate. The overnight cultures (ONCs) were incubated at 30 °C and 120 rpm.

## 6.4. Preparation of whole cell systems

0.3 L LB medium supplemented with ampicillin (100 µg/mL) were inoculated with 3 mL of the previously prepared over night culture and shaken at 30 °C and 120 rpm until the cell density reached an $OD_{600}$ of 0.6. At this point expression was induced with the appropriate inducer [pet21a(+) and pet16b(+): isopropyl-*β*-D-thiogalactopyranoside (IPTG, 300 µL, 238 mg/ml $H_2O_{dist.}$), pask-IBA35(+): Anhydrotetracyclin (ATC, 300 µL, 2 mg/mL ethanol)]. The cultures were grown at 20 °C and 120 rpm overnight. Subsequent centrifugation (8000 rpm, 20 min, 4 °C), washing with phosphate buffer (100 mM, pH 7.0), centrifugation (8000 rpm, 20 min, 4 °C) and lyophilisation of the cell pellet gave a whole cell preparation which was used for all experiments.

**Table 15: List of enzymes overexpressed in *E. coli* BL21 (DE3) and used as whole cell preperation**

| Enzyme | Organism | Plasmid vector | Inducer |
|---|---|---|---|
| ω-transaminase | *Vibrio fluvialis* | pASK-IBA35(+) | ATC |
| ω-transaminase | *Chromobacterium violaceum* | pET21a(+) | IPTG |
| long chain alcohol oxidase | *Aspergillus fumigatus* | pET21a(+) | IPTG |
| pyranose oxidase | *Trametes ochracea* | pET21a(+) | IPTG |
| pyranose oxidase | *Phanerochaete chryosporium* | pET16b(+) | IPTG |
| gulonolactone oxidase | *Lysinibacillus spaericus* | pET21a(+) | IPTG |
| alditol oxidase | *Streptomyces coelicolor* | pET21a(+) | IPTG |

| | | | |
|---|---|---|---|
| hexose oxidase | *Chrondus crispus* | pET21a(+) | IPTG |
| glycerol-3-phosphate oxidase | *Rhodoccocus jostii* | pET21a(+) | IPTG |
| alanine dehydrogenase | *Bacillus subtilis* | pASK-IBA35(+) | ATC |

## 6.5. Preparation of empty host cells

0.3 L LB medium was inoculated with 3 mL of a previously prepared over night culture (ONC) and were grown at 30 °C, 120 rpm overnight. Subsequent centrifugation (8000 rpm, 20 min, 4 °C), washing with phosphate buffer (pH 7.0, 100 mM), centrifugation (8000 rpm, 20 min, 4 °C) and lyophilisation of the cell pellet gave a whole cell preparation which was used for all experiments.

## 6.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The separating gel was prepared by mixing acrylamide (5 mL, 30 w/v) with separating gel buffer (5.625 mL, 1 M Tris/HCl, pH 8.8), distilled water (4.093 mL), sodium dodecyl sulphate (SDS) stock solution in distilled water (150 µL, 10 %), ammonium persulfate in distilled water (APS, 120 µL, 10 %) and *N*,*N*,*N*',*N*'-tetramethylethylendiamine (TEMED, 12 µL). The separating gel was poured in a gel caster, whereby it polymerised. Subsequently, a thin layer of isopropanol was added, so that an even surface on top of the separating gel surface was formed. The isopropanol was removed after polymerisation. Next, the stacking gel was prepared by mixing acrylamide (0.833 mL, 30 %) with stacking gel buffer (0.625 mL, 1 M Tris/HCl, pH 6.8), distilled water (3.462 mL), SDS stock solution in distilled water (50 µL, 10 %), APS stock solution in distilled water (25 µL, 10 %) and TEMED (5 µL) to induce the polymerisation. The stacking gel was poured in the gel caster on top of the separating gel. A comb that was placed to create wells was removed after polymerisation of the gel.

## 6.7. General procedure for the expression and purification of the alanine dehydrogenase

For overexpression of L-alanine dehydrogenase from *Bacillus subtilis* in *E. coli*, an ONC was prepared to inoculate the main culture (LB/Amp media- 12 L in total). The cells were grown at 37 °C and 180

rpm to an $OD_{600}$ of 0.6. The protein expression was induced by addition of anhydrotetracyclin (ATC, 100 µL, 2 mg/mL solution in ethanol) and the cells were incubated at 30 °C and 180 rpm for 3 hours. The cells were harvested by centrifugation (8000 rpm, 15 minutes, 4 °C). The cell pellet was washed with phosphate buffer (100 mM, pH 7.0) and frozen overnight. The cells were disrupted using ultra-sonication (pulse 1 s, pause 4 s, 8 minutes, 40 % amplitude) followed by centrifugation (18000 rpm, 20 minutes, 4 °C). The supernatant was collected and purified with a His-prep-column.

The column was washed with distilled water for 10 minutes and then equilibrated with washing buffer (100 mM phosphate buffer, pH 7.0, 10 mM imidazole). The crude enzyme preparation was loaded onto the column which was then eluted with binding-buffer (100 mM, pH 7.0 phosphate buffer, 20 mM imidazole). After removal of unspecific proteins, the elution was performed with phosphate buffer (100 mM, pH 7.0, 300 mM imidazole) and the eluted alanine dehydrogenase fractions were collected in 15 mL Sarstedt tubes (flow 2.00 mL/min). The purity of the collected fractions was checked using SDS-page (Figure 5).

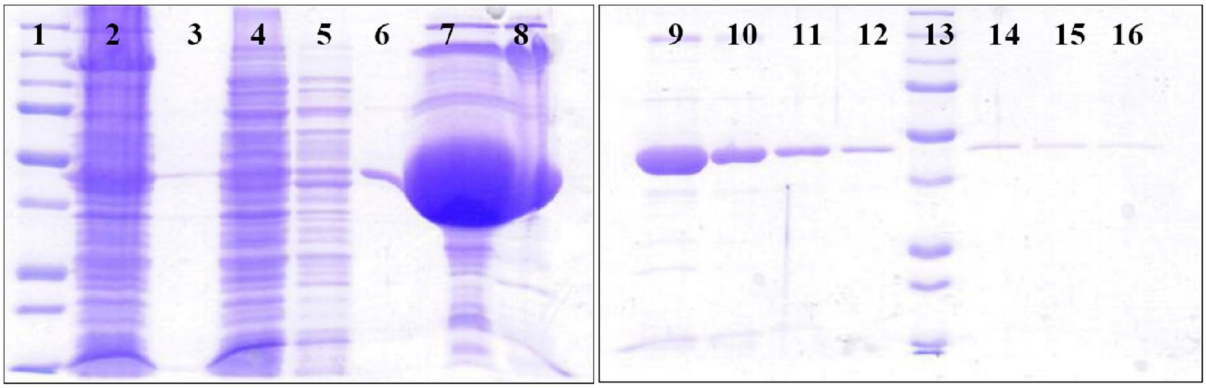


**Figure 5: SDS-gel of the His-Tag purification process: lane 1 &13: Precision Plus Protein All Blue Standard, lane 2: crude extract, lane 3: pellet, lane 4: wash fraction 1, lane 5: wash fraction 2, lane 6-12, 14-16: Ala-DH containing fractions**

The pure fractions were combined and desalted by dialysis overnight at 4 °C against phosphate buffer (pH 7.0, 5 L, 100 mM). Finally, the desalted enzyme was concentrated with Satorius Stedim biotech centrifugation tubes (10 × 103 kDa), aliquoted and stored at -20 °C.

## 6.8. Crude ω-transaminase preparation

ω-Transaminases were overexpressed in *E. coli* BL21 (DE3). Lyophilised cells (250 mg, dry weight) were suspended in phosphate buffer (7.0 pH, 4 mL, 100 mM) containing PLP (1 mM) and EDTA (1 mM) as well as lysozyme from chicken egg white (2.5 mg, lyophilised powder, 95 % protein, >40000

U/mg) and the protease inhibitor phenylmethanesulfonyl fluoride (PMSF, 1 mM). The lysis was performed while shaking (170 rpm) at rt for 3 h. The mixture was centrifuged and the supernatant was aliquoted and lyophilised.

## 6.9. Crude long chain alcohol oxidase preparation

Long chain alcohol oxidase (*A. fumigatus*) were overexpressed in *E. coli* BL21 (DE3). Lyophilised cells (250 mg) were suspended in phosphate buffer (pH 7.0, 4 mL, 100 mM) containing 1 mg/mL FAD. The suspension was ultra-sonicated at 0 °C for 2.5 min with 40 % amplitude, 1 s pulse and 4 s pause (Branson Digital sonifier®). The disrupted cells were centrifuged, the pellet separated and the supernatant aliquoted and stored at 4 °C and -20 °C.

## 6.10. Amino acid sequences

### 6.10.1. Long chain alcohol oxidase from *Aspergillus fumigatus*

MAEQAVTAYVPLDVPLPPIPEGQVFSDLQWRTLLSLADTVIPSIRSTSLPKSVSTKVVPESTFKDAVSTLASHI
HDPDATQIAEQYLEENASANPQFVEGLRRLFAEYIHEEGKSGINLILNALNSKAGSLILTGSTTPIQDQPFEIR
EKIFSSWETSRIKPLRAIYRAFTAIFKKTWTVSPTIRSVVGCPRVPIHGKPADGFEYEFLQFPPGAEPETIDTD
VVIVGSGCGGSVAAKLAEAGYRVLVVEKSYHYPSKYFPMDFNEGFVSMFENGGATTSDDGSIAVLAGSTWGG
GGTVNWSASLQTQGYVRREWASKGLPFFESHEYQQALDRVCDRMGVSNDHTEHNYSNRVLLDGARKLGY
AAQPVPQNTGGSNHYCGYCTMGCHSCGKKGPRETFLADAAKAGTTFIEGFRADKIRFKNTKGGRVACGVEG
TWTSRDSYLGTAGPDRTTRKVIINASKVIVSCGTLHSPLLLLRSGLKNPQIGRNLYLHPVVLSCAVFDEEIRP
WEGSALTIVVNEFEDQDGQGHGVKIENVVMLPALYLPTFPWRDGLDYKLWAAKLPRMSGFIALTKERDAG
RVYPDPADGRVRIDYTVSAYDRKHIVEALIATAKIAYISGAREFHTSNREMPPFIRPTEASDPNAPEGVTNQA
LQAWIAVLRRKNPVDPERTQYASAHQMGTCRMGSSPRTSVVDPECQVWGTQGLYVMDASVFPSASGVNP
MVTNMAIADWASRKVVRSLEKANHGKTVLARL

### 6.10.2. ω-Transaminase from *Chromobacterium violaceum* DSM 30191

MQKQRTTSQWRELDAAHHLHPFTDTASLNQAGARVMTRGEGVYLWDSEGNKIIDGMAGLWCVNVGYGR
KDFAEAARRQMEELPFYNTFFKTTHPAVVELSSLLAEVTPAGFDRVFYTNSGSESVDTMIRMVRRYWDVQG
KPEKKTLIGRWNGYHGSTIGGASLGGMKYMHEQGDLPIPGMAHIEQPWWYKHGKDMTPDEFGVVAARWL
EEKILEIGADKVAAFVGEPIQGAGGVIVPPATYWPEIERICRKYDVLLVADEVICGFGRTGEWFGHQHFGFQP

DLFTAAKGLSSGYLPIGAVFVGKRVAEGLIAGGDFNHGFTYSGHPVCAAVAHANVAALRDEGIVQRVKDDIG
PYMQKRWRETFSRFEHVDDVRGVGMVQAFTLVKNKAKRELFPDFGEIGTLCRDIFFRNNLIMRACGDHIVS
APPLVMTRAEVDEMLAVAERCLEEFEQTLKARGLA

### 6.10.3. ω-Transaminase from *Vibrio fluvialis*

MASRGSHHHHHHGANKPQSWEARAETYSLYGFTDMPSLHQRGTVVVTHGEGPYIVDVNGRRYLDANSGL
WNMVAGFDHKGLIDAAKAQYERFPGYHAFFGRMSDQTVMLSEKLVEVSPFDSGRVFYTNSGSEANDTMVK
MLWFLHAAEGKPQKRKILTRWNAYHGVTAVSASMTGKPYNSVFGLPLPGFVHLTCPHYWRYGEEGETEEQ
FVARLARELEETIQREGADTIAGFFAEPVMGAGGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWG
CVTYDFTPDAIISSKNLTAGFFPMGAVILGPELSKRLETAIEAIEEFPHGFTASGHPVGCAIALKAIDVVMNEG
LAENVRRLAPRFEERLKHIAERPNIGEYRGIGFMWALEAVKDKASKTPFDGNLSVSERIANTCTDLGLICRPL
GQSVVLCPPFILTEAQMDEMFDKLEKALDKVFAEVA

### 6.10.4. Alanine dehydrogenase from *Bacillus subtilis*

MASRGSHHHHHHIIGVPKEIKNNENRVALTPGGVSQLISNGHRVLVETGAGLGSGFENEAYESAGAEIIADPK
QVWDAEMVMKVKEPLPEEYVYFRKGLVLFTYLHLAAEPELAQALKDKGVTAIAYETVSEGRTLPLLTPMSE
VAGRMAAQIGAQFLEKPKGGKGILLAGVPGVSRGKVTIIGGGVVGTNAAKMAVGLGADVTIIDLNADRLRQL
DDIFGHQIKTLISNPVNIADAVAEADLLICAVLIPGAKAPTLVTEEMVKQMKPGSVIVDVAIDQGGIVETVDHI
TTHDQPTYEKHGVVHYAVANMPGAVPRTSTIALTNVTVPYALQIANKGAVKALADNTALRAGLNTANGHV
TYEAVARDLGYEYVPAEKALQDESSVAGA

## 6.11. Screening procedures

### 6.11.1. Optimised screening procedure for the cascade system

Whole cell preparations of ω-transaminase from *C. violaceum* (20 mg, lyophilised dry weight) and long chain alcohol oxidase (40 mg, lyophilised dry weight) were each resuspended in sodium phosphate buffer (500 µL, 100 mM, pH 10.0) supplemented with PLP (2 mM), $NAD^+$ (2 mM) and $FAD^+$ (1 mg/mL) and were shaken at 30 °C and 120 rpm for 30 min in a horizontal position. A solution of L-alanine (13 mg, 0.15 mmol), ammonium chloride (5.3 mg, 0.1 mmol) and glucose (21.6 mg, 0.12 mmol) in sodium phosphate buffer (500 µL, 100 mM, pH 10.0) were added. 10 µL alanine dehydrogenase from *B. subtilis* (7.5 mg protein/mL stock solution), 20 µL glucose dehydrogenase

(GDH 0.001 DSM 7 U/mg $NAD^+$, 20 mg/mL stock solution) and 10 µL catalase from *M. lysodeikticus* (170000 U/mL) were added. Then 10 mM substrate was added and the reaction mixture was placed into the oxygen apparatus. The apparatus was primed with oxygen (technical grade for about 1 min and pressurised to 2 bar). The whole apparatus was shaken at rt and 170 rpm for 24 h (MG5 vials in vertical position). For derivatisation, ethyl(succinimidooxy)formate (60 mg, 0.33 mmol) dissolved in acetonitrile (500 µL) was added to the reaction mixture (10 mM substrate loading) fllowed by the addition of triethylamine (100 µL, 10 % v/v). The mixture was shaken at 45 °C and 500 rpm for 1 hour. Afterwards, the mixture was extracted twice with EtOAc (500 µL). The combined organic phases were dried over $Na_2SO_4$.

### 6.11.2. Initial cascade conditions

Whole cell preparations of ω-transaminase from *V. fluvialis*[a] (20 mg, lyophilised dry weight) and long chain alcohol oxidase[b] (20 mg, lyophilised dry weight) were each resuspended in sodium phosphate buffer (pH 10.0, 500 µL, 100 mM) supplemented with PLP (2 mM), $NAD^+$ (2 mM), $FAD^+$ (1 mg/mL) and were shaken at 30 °C and 120 rpm for 30 min in a horizontal position. A solution of L-alanine (13 mg, 0.15 mmol), ammonium chloride (5.3 mg, 0.1 mmol) and glucose (21.6 mg, 0.12 mmol) in sodium phosphate buffer (500 µL, 100 mM, pH 7.0) were added. 10 µL alanine dehydrogenase from *B. subtilis* (7.5 mg protein/mL stock solution), 20 µL glucose dehydrogenase (GDH 0.001 DSM 7 U/mg $NAD^+$, 20 mg/mL stock solution) and 15 µL HRP/ABTS (10 mg/mL each) were added. Then 33.3 mM substrate was added and the reaction mixture was placed into the oxygen apparatus. The apparatus was primed with oxygen (technical grade) for about 1 min and pressurised to 4 bar. The whole apparatus was shaken at rt and 170 rpm for 20 h (MG5 vials in vertical position). For derivatisation, ethyl(succinimidooxy)formate (60 mg, 0.33 mmol) dissolved in acetonitrile (500 µL) was added to the reaction mixture (33.3 mM substrate loading). Triethylamine (100 µL, 10 % v/v) was added. The mixture was shaken at 45 °C and 500 rpm for 1 hour. Afterwards, the mixture was extracted twice with EtOAc (500 µL). The combined organic phases were dried over $Na_2SO_4$.

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[a] ω-Transaminase from *C. violaceum* and *P. denitrificans* were used under these conditions.

[b] Alternativly pyranose oxidase from *T. ochracea* whole cells (20 mg, lyophilised dry weight); phosphate buffer (pH 7.0, 100 mM)

### 6.11.3. Screening of flavin dependend oxidase

Whole cell preparations of oxidase (20 mg lyophilised dry wheight) was suspended in phosphate buffer (1 mL, 100 mM, pH 7.0, 1 mg/mL) and were shaken at 30 °C and 120 rpm for 30 min in a horizontal position. 30 µL HRP/ABTS assay (each 10 mg/mL) was added. Afterwards 25 mM substrate was added and the reaction mixture was placed into the oxygen apparatus. The apparatus was primed with oxygen (technical grade for about 1 min and pressurised to 1 bar). The whole apparatus was shaken at rt and 170 rpm for 20 h (MG5 vials in vertical position).

### 6.11.4. Screening of ω-transaminase

Whole cell preparations of ω-transaminase (20 mg lyophilised dry wheight) was suspended in phosphate buffer (500 µL, 100 mM, pH 7.0) supplemented with PLP (2 mM), $NAD^+$ (2 mM) and were shaken at 30 °C and 120 rpm for 30 min in a horizontal position. A solution of L-alanine (13 mg, 0.15 mmol), ammonium chloride (5.3 mg, 0.1 mmol) and glucose (21.6 mg, 0.12 mmol) in sodium phosphate buffer (500 µL, 100 mM, pH 10.0) were added. 10 µL alanine dehydrogenase from *B. subtilis* (7.5 mg protein/mL stock solution), 20 µL glucose dehydrogenase (GDH 0.001 DSM 7 U/mg $NAD^+$, 20 mg/mL stock solution) were added. Then 50 mM substrate was added and the reaction mixture was shaken at 30 °C and 120 rpm overnight at horizontal position. For derivatisation, ethyl(succinimidooxy)formate (60 mg, 0.33 mmol) dissolved in acetonitrile (500 µL) was added to the reaction mixture followed by the addition of triethylamine (100 µL, 10 % v/v). The mixture was shaken at 45 °C and 500 rpm for 1 hour. Afterwards, the mixture was extracted thrice with dichlormethane (500 µL). The combined organic phases were dried over $Na_2SO_4$.

### 6.11.5. Screening procedure for cell free extract of long chain alcohol oxidase

The cell free extract of long chain alcohol oxidase from *A. fumigatus* (250 µL, supernatant/resuspended pellet) and ω-transaminase from *V. fluvialis* (10 mg, lyophilised dry weight) was suspended in phosphate buffer (250 µL, 100 mM, pH 7.0, 2 mM PLP, 2 mM $NAD^+$) and were shaken at 30 °C and 120 rpm for 30 min in a horizontal position. A solution of L-alanine (7.5 mg, 0.15 mmol), ammonium chloride (2.6 mg, 0.1 mmol) and glucose (10.5 mg, 0.6 mmol) in phosphate buffer (250 µL, 100 mM, pH 7.0) were added. 5 µL alanine dehydrogenase from *B. subtilis* (7.5 mg protein/mL stock solution), 10 µL glucose dehydrogenase (GDH 0.001 DSM 7 U/mg $NAD^+$, 20 mg/mL stock solution) were added. 15 µL HRP/ABTS stock solution [10 mg/mL in phosphate

buffer (pH 7.0, 100 mM)] was added. Then 33.3 mM substrate was added and the reaction mixture was placed into the oxygen apparatus. The apparatus was primed with oxygen (technical grade for about 1 min) and placed in a thermoshaker and was shaken at rt and 170 rpm for 20 h. The mixture was derivatised as described before.

## 6.12. Screening procedure for surface influence

The general cascade-procedure was performed. The mixture was put in MG5 vial and a 10 mL flask. The vial and the flask were placed into the oxygen apparatus. The oxygen apparatus was pressurised with 3 bar $O_2$ technical grade. The apparatus was shaken as described for 20 h.

## 6.13. Applying the ABTS/HRP assay

Instead of the catalase from *M. lysodeikticus* (170000 U/mL) a horseradish peroxidase/2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) stock solution (30 µL) was added to the mixture. The stock solution containing HRP (10 mg) and ABTS (10 mg) dissolved in 1 mL phosphate buffer (pH 7.0, 100 mL).

## 6.14. Different methods of oxygen supply

1) The reaction vials were placed in an oxygen apparatus. The apparatus was primed with technical oxygen (technical grade for about 1 min) and then pressurised to the desired pressure at rt.

2) The reaction vials were primed with oxygen, then closed and placed either in an oxygen apparatus pressurised with 2 bar or placed under atmospheric conditions at rt.

3) The reaction vials were closed with paraffin film which was pierced and placed under atmospheric condition at rt.

## 6.15. Preparation of phosphate buffer for pH study

Phosphate buffer (pH 7.0, 100 mM) was prepared and adjusted to the desired pH (pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, pH 10.0, pH 11.0 and pH 12.0) with phosphoric acid (conc.) and NaOH (conc. and 1 M).

## 6.16. Derivatisation protocols for sample splitting

### 6.16.1. Derivatisation of the amine

1) Ethyl(succinimidooxy)formate (60 mg, 320 µmol) were dissolved in acetonitrile (500 µL) was added to the supernatant of the reaction mixture which was split before in two parts (2 × 750 µL). 100 µL 10 % v/v triethylamine was added. The mixture was shaken at 45 °C and 500 rpm for 1 hour. Afterwards, the mixture was extracted twice with EtOAc (500 µL). The combined organic phases were dried over $Na_2SO_4$.

### 6.16.2. Derivatisation of the acid

2) The reaction mixture was dried in the SpeedVac according to suppliers protocol. 500 µL MeOH and catalytic $H_2SO_4$ (conc.) was added. The mixture was shaken at 60 °C and 500 rpm. The solvent was removed and $H_2O$ (500 µL) were added. The mixture was extracted thrice with EtOAc (500 µL). The combined organic phases were dried over $Na_2SO_4$.

## 6.17. Synthesis and GC-MS analysis

### 6.17.1. Ethyl(succinimidooxy)formate

A solution of *N*-hydroxysuccinimide (5.7 g, 50 mmol, 1.0 eq.) in dry THF (80 mL) was placed in a 250 mL three-neck flask equipped with a condenser, a magnetic stirrer, and a pressure-equalising additional funnel. The contents were cooled on an ice bath and ethylchloroformate (7.6 g, 70 mmol, 1.4 eq.) was added dropwise over 5 min followed by dry THF (10 mL). The mixture was stirred and triethylamine (10.4 mL, 75 mmol, 1.5 eq.) in dry THF (10 mL) was added dropwise over 10 min with continous stirring. A thick white precipitate was formed and the reaction mixture was allowed to warm up to room temperature with stirring for 2 h. The reaction mixture was filtered and the solvent was removed under reduced pressure to give a reddish-brown residue. Recrystallisation from ethanol afforded white glassy crystals.

Yield = 4.91 g (52 %, white crystals)

$^{1}$H-NMR (300 MHz, $CDCl_3$) $\delta$ [ppm] = 1.39 (t, 3H, $J$ = 9.0 Hz, $CH_3$), 2.83 (s, 4H, succinimido ring), 4.39 (dd, 2H, $J$ = 9.0 Hz; $J$ = 15.0 Hz, $CH_2$).

$^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ [ppm] = 13.7, 25.2, 67.3, 151.2, 168.4.

### 6.17.2. Retention time

The analytical method for GC-MS analysis was described in the general section. Hereafter are the retention times for the substrates, intermediates and products.

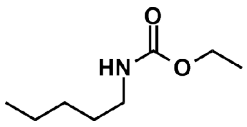
**Table 16: Retention times of analysed compounds**

| $t_R$ [min] | Alcohol | Aldehyde | Derivatised amine | Acid |
|---|---|---|---|---|
| 1-hexanol | 5.74 | 4.90 | 9.61 | 6.80 |
| benzyl alcohol | 7.42 | | 11.05 | |
| 6-chlorhexan-1-ol | 8.18 | | 11.78 | |

| | | | |
|---|---|---|---|
| 8-chloroctan-1-ol | 9.79 | 13.19 | |
| 6-bromhexan-1-ol | 8.85 | 12.43 | |
| 8-bromoctan-1-ol | 10.53 | 13.36 | |
| 1-pentanol | 4.51 | 8.78 | |
| 1-heptanol | 6.69 | 10.36 | |
| 1-octanol | 7.90 | 11.16 | |
| 1-nonanol | 8.43 | 11.93 | 9.07 |

## 6.17.3. Products of biotransformation and derivatisation

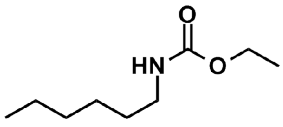
**ethyl *N*-pentylcarbamate**




$t_R$ = 8.78

*m/z*= 159 (10 %), 130 (5%), 102 (100 %), 90 (10 %), 74 (10 %), 58 (10 %), 43 (15 %), 30 (70 %).

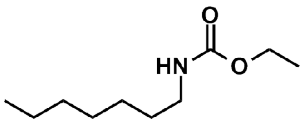
**ethyl *N*-hexylcarbamate**




$t_R$ = 9.61

*m/z* = 173 (10 %), 144 (10 %), 102 (100 %), 74 (10 %), 55 (10 %), 30 (40 %).

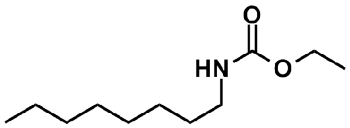
**ethyl *N*-heptylcarbamate**




$t_R$ = 10.36

*m/z* = 187 (5 %),158 (10 %), 102 (100 %), 90 (15 %), 74 (10 %), 57 (10 %), 41 (10 %), 30 (40 %).

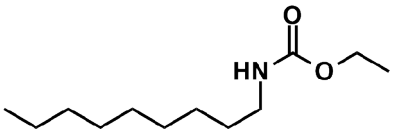
**ethyl *N*-octylcarbamate**




$t_R$ = 11.16

*m/z* = 201 (5 %), 172 (10 %), 102 (100 %), 90 (20 %), 77 (20 %), 55 (10 %), 41 (15 %), 30 (40 %).

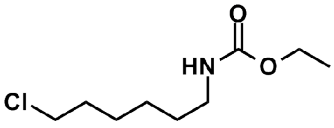
**ethyl *N*-nonylcarbamate**




$t_R$ = 11.93

*m/z* = 215 (5 %), 186 (10 %), 102 (100 %), 90 (20 %), 74 (10 %), 55 (10 %), 41 (15 %), 30 (40 %).

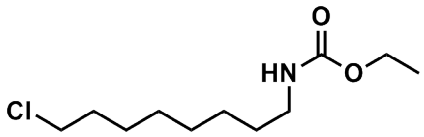
**ethyl *N*-(6-chlorohexyl)carbamate**




$t_R$ = 11.78

*m/z* = 207 (5 %), 172 (15 %), 102 (100 %), 90 (5 %), 74 (5 %), 55 (10 %), 41 (10 %), 30 (40 %).

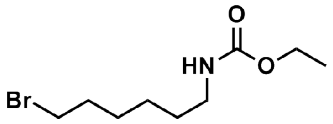
**ethyl *N*-(8-chlorooctyl)carbamate**




$t_R$ = 13.19

*m/z* = 235 (5 %), 200 (15 %), 102 (100 %), 90 (15 %), 69 (10 %), 55 (15 %), 30 (30 %).

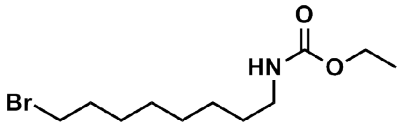
**ethyl *N*-(6-bromohexyl)carbamate**




$t_R$ = 12.43

*m/z* = 253 (<1 %), 251 (<1 %), 172 (30 %), 102 (100%), 83 (5 %), 55 (15 %), 41 (15 %), 30 (40 %).

**ethyl *N*-(8-bromooctyl)carbamate**




$t_R$ = 13.36

*m/z* = 281 (5 %), 200 (25 %), 154 (5 %), 129 (5 %), 102 (100 %), 90 (15 %), 69 (10 %), 55 (15 %), 30 (40 %).

## Abbreviations

| | |
|---|---|
| 4-AAP | 4-aminoantipyrine |
| AAO | aryl alcohol oxidase |
| ABTS | 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) |
| *A. cellulolyticus* | *Acidothermus cellulolyticus* |
| ADH | alcohol dehydrogenase |
| ALS | acetolactate synthase |
| Amp | Ampicillin |
| *A. niger* | *Aspergillus niger* |
| APS | ammonium persulfate |
| ATC | anhydrotetracyclin |
| *A. terreus* | *Aspergillus terreus* |
| *A. thaliana* | *Arabidopsis thaliana* |
| *A. viridans* | *Aerococcus viridans* |
| *B. sterolicum* | *Brevibacterium sterolicum* |
| CataCXiumPCy | 2-(Dicyclohexylphosphino)-1-phenyl-1*H*-pyrrole |
| *C. boidinii* | *Candida boidinii* |
| *C. cloacae* | *Candida cloacae* |
| *C. crispus* | *Chrondus crispus* |
| CHAPS | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| conc. | concentrated |
| conv. | conversion |
| *C. vicina* | *Calliphora vicina* |
| *C. violaceum* | *Chromobacterium violaceum* |
| *C. tropicalis* | *Candida tropicalis* |
| DMSO | dimethyl sulfoxide |
| EC | enzyme commission number |
| FAD | flavin adenine dinucleotide |
| *F. meningosepticum* | *Flavobacterium meningosepticum* |
| FMN | flavin mononucleotide |
| *E. coli* | *Escherichia coli* |

| | |
|---|---|
| *ee* | *enantiomeric excess* |
| EI | electron ionisation |
| eq. | equivalents |
| GDH | glucose dehydrogenase |
| GOase | galactose oxidase |
| *G. sepiarium* | *Gloeophyllum sepiarium* |
| HRP | horseradish peroxidase |
| HOAX | (*S*)-2-hydroxy acid oxidase |
| HOX | hexose oxidase |
| IPTG | isopropyl-*β*-D-thiogalactopyranoside |
| LB | Luria-Bertani |
| LCAO | long chain alcohol oxidase |
| NADH | nicotinamide adenine dinucleotide |
| n.c. | no conversion |
| n.q. | no quantified |
| $OD_{600}$ | optical density at λ= 600 nm |
| ONC | overnight culture |
| *P. eryngii* | *Pleurotus eryngii* |
| *P. gigantea* | *Peniophora gigantea* |
| *P. chryosporium* | *Phanerochaete chryosporium* |
| PLP | pyridoxal phosphate |
| PMP | pyridoxamine |
| *P. ostreatus* | *Pleurotus ostreatus* |
| *P. pastoris* | *Pichia pastoris* |
| *P. putida* | *Pseudomonas putida* |
| PSA | phenol-4-sulfonic acid |
| *P. simplicissimum* | *Penicillium simplicissimum* |
| PVA | poly(vinylalcohol) |
| *P. vesicularis* | *Pseudomonas vesicularis* |
| rt | room temperature |
| SCAO | short chain alcohol oxidase |
| *S. coelicolor* | *Streptomyces coelicolor* |
| SDS | sodium dodecyl sulphate |
| *S. hygroscopicus* | *Streptomyces hygroscopicus* |

| | |
|---|---|
| *Sp. oleracea* | *Spinacia oleracea* |
| *T. aurantiacus* | *Thermoascus aurantiacus* |
| TEA | triethylamine |
| TEMED | *N*,*N*,*N*',*N*'-tetramethylethylendiamine |
| THF | tetrahydrofuran |
| *T. hirsuta* | *Trametes hirsuta* |
| *T. matsutake* | *Tricholoma matsutake* |
| *T. ochracea* | *Trametes ochracea* |
| $t_R$ | retention time |
| α-TA | α-transaminase |
| ω-TA | ω-transaminase |
| U | enzyme activity [1 U= 1 µmol/min] |
| v/v | volume/volume |
| w/v | weight/volume |

# CURRICULUM VITAE

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