Biocatalytic conversions by white-rot funghi: exploting teh reductive enzyme system

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Chapter 1

General Introduction

Traditionally, the industrial production of most organic compounds has been carried out by using organic synthesis. Present methods are still viable, but in several cases bacterial or fungal enzymes can catalyze reactions that are difficult or, at present time, impossible to emulate using other techniques of organic chemistry.

Bacteria and fungi colonize virtually every ecological niche. In order to survive they carry out metabolic processes, utilizing whatever nutrients are available in their particular environment, interconverting diverse organic compounds. Consequently, micro-organisms are able to catalyze an enormous variety of chemical reactions that transform both naturally occurring and human-made organic compounds. These biotransformations occur with high specificity and efficiency, mainly because they are catalyzed by enzymes (Glazer and Nikaido 1995).

Enzymes lower the activation energy to the point where reactions can be performed at room temperature or even lower. Few chemical agents can match the specificity that enzymes achieve under mild aqueous conditions. Furthermore, they can accelerate the rates of chemical reactions by factors of 10^8 to 10^{12}. Even when an organic compound *can* be synthesized chemically, the process may require many steps where a single enzyme-catalyzed reaction can often achieve the same end (Glazer and Nikaido 1995). Enzymes can be used as biocatalysts, either in the form of (partially) purified enzyme or as a part of a whole-cell system (micro-organism).

Several factors have strengthened the impact of enzyme-catalyzed reactions in organic chemistry over the past 15 years:

1) A wide range of enzymes is now commercially available.
2) The chemo-, regio- and stereoselectivity of the reactions catalyzed by various enzymes are much better understood.
3) Relatively mild reaction conditions of temperature and pH can be used, a particular advantage when the desired product is rather labile.
4) Pharmaceutical, agricultural, flavor and fragrance industries are increasingly committed to provide their products in enantiomerically pure form, requiring new production methods.
5) The increasing consumer demand for natural foods creates opportunities for enzymes used in the production of food additives or even enzymes used as novel food additives (modified from Wilson and Walker 1991).

Consequently, biocatalysts are being used increasingly in industry to carry out important chemical transformations. Enzymes or whole cell systems are currently used as biocatalysts in the manufacture of a wide variety of substrates, including steroids, semisynthetic antibiotics, carbohydrate derivatives and amino acids and enantiomerically pure acids esters, alcohols and amines (Liese *et al.* 2000; Koeller and Wong 2001; Schmid *et al.* 2001).

Although biotransformations of natural compounds can be economically attractive for application in industry, and profits of naturally derived products may exceed the profits made from chemically based products, adjustments or replacements of existing chemical procedures are generally not favored by industry due to initial increased expenditures. To compete with commonly used chemical methods, new processes must be highly efficient and thorough knowledge and technological experience is required.

Biocatalytic conversions which are potentially more efficient, and therefore challenging, are both the chemically cumbersome reductions of carboxylic acids and the asymmetric reduction of ketones.

Acid reduction

Acid reduction, yielding aldehydes and/or alcohols, is of great interest to industry. Fields of application can be found in the production of both pharmaceuticals and food and flavor compounds. Many acid reductions with potential industrial relevance exist. One example is the production of flavor compounds. Vanillic acid can be reduced to vanillin and vanillyl alcohol (Lesage-Meessen *et al.* 1997). Also the abundant phenolic compound ferulic acid can be used as starting material for vanillin (Rosazza *et al.* 1995).

Reduction of carboxylic acids to their corresponding aldehydes and alcohols is an energetically difficult reaction. To overcome the activation energy needed, a redox potential of at least –600mV is required (Thauer *et al.* 1977).

One traditional method of producing aldehydes via reduction of acids is the Rosenmund reduction (Rosenmund 1918). First the (aromatic) acid is converted to the corresponding acid-chloride, followed by conversion into the aldehyde via reduction with hydrogen gas over a suitable (Pd) catalyst.

Direct chemical reduction of carboxylic acids was made possible in 1946 by the discovery of the powerful reducing agent lithium aluminium hydride ($LiAlH_4$) (Solomons 1988). Several catalysts have been developed for the direct chemical reduction of carboxylic acids since. In most cases, the high temperatures and high hydrogen pressures that are required for the reaction make the processes less suitable for industrial applications. Reactions that may proceed at lower temperatures are reductions using a zirconium-titaniumoxide catalyst and an alcohol as hydrogen source (Fukumoto and Yamamoto 1988), or di-isobutyl aluminium hydride as the reductant in the presence of a specific alkyl aluminium chloride (Iwao and Uchimiya 1997; E.C.D. van den Ban, pers. comm.)

BIOCATALYTIC ACID REDUCTION

Although reductions of carboxylic acids to aldehydes or alcohols energetically difficult reactions, numerous micro-organisms are capable of acid reduction. Both aliphatic and aromatic acids are enzymatically reduced by diverse bacteria (*e.g. Acinetobacter, Clostridium, Mycobacterium* and *Nocardia* species), fungi (*Actinomyces, Aspergillus, Bjerkandera, Glomerella, Phanerochaete, Pycnoporus* and *Trametes*) (Farmer and Henderson 1959; Kato *et al.* 1990; Arfmann and Abraham

1993) and archeae (*Pyrococcus* and *Thermococcus*) (Heider *et al.* 1995; van den Ban *et al.* 1999).

In analogy with the chemical methods described above, two different ways exist for the biocatalytic reduction of a carboxylic acid: with or without activation of the acids. Activation of acids may proceed via either acyl-AMP (Zenk and Gross 1965) or acyl-CoA (Rodriguez *et al.* 1983). Although to the best of our knowledge activation of acids to acyl-CoA has not been described yet for white-rot fungi, the subject of this thesis, reduction of this acyl-CoA has been described for some bioluminescent bacteria (Rodriguez and Meighen 1984; Wall *et al.* 1985).

Aryl aldehyde oxidoreductases both catalyze the ATP-dependent activation of the acid and the NADPH-dependent reduction of acyl-AMP. In case the acid has not been activated, a cofactor is required. Which cofactor is needed is yet unknown, but artificial cofactors such as methylviologens can be used (Huber *et al.* 1995). The active sites of these enzymes usually contain tungsten or molybdenum (White *et al.* 1991; White *et al.* 1993; E.C.D. van den Ban, pers. comm.).

Advantages of biocatalytic acid reductions are the mild reaction conditions that are usually used and the high specificity and selectivity of the enzymatic reactions. Furthermore, especially for food and flavor components, products with the label 'natural' can have a substantially higher value than the same products that are chemically produced, owing to the consumer demand. Legislation allows the origin of natural to vary from the compound derived directly from natural sources, to the production by cultured cells from e.g. fungi and bacteria (Rosazza *et al.* 1995; Lesage-Meessen 1997), or even incubation mixtures in which only enzymes are applied (Markus *et al.* 1992).

A drawback of biocatalytic systems is that most of the catalysts reduce the aldehydes further to the alcohols, whereas in some cases the aldehydes are more valuable than alcohols. Therefore, novel approaches to produce aldehydes (or alcohols) from carboxylic acids using bioconversion are highly desirable.

Asymmetric reduction of ketones for the production of optically active alcohols

Many naturally occurring compounds exist as one of two possible enantiomers. The chirality of living beings, which is reflected at the most fundamental level in the stereochemistry of enzyme systems and other proteins, requires that chemical reactions take place with a particular orientation in space. Therefore chirality of compounds can have determining effects on its interaction with living organisms. Whereas one enantiomer of a compound may be active as a medication or may be a hormone, its mirror image may have the exact opposite effect or be biologically inactive. For example: the dextrorotatory (+) isomer of the antitubercular ethambutol 2,2'-(ethylenediimino)-di-1-butanol dihydrochloride has potent antitubercular activity, whereas the opposite (-) enantiomer causes degeneration of the optic nerve, leading to blindness (Glazer and Nikaido 1995). This sensitivity of biological systems to chirality is one of the main concerns in the application of synthetic compounds as pharmaceuticals, agrochemicals and food additives. In recent years, regulations on the marketing of new drugs have become stricter with respect to chiral compounds. Licenses for the use of racemic mixtures as new active ingredient are only granted if the activity of both enantiomers has been established and the evidence that the unwanted enantiomer does not cause any adverse effects has been produced (de Camp 1989).

Chiral alcohols form an important class of intermediates for fine chemicals and pharmaceuticals. There are several well-established routes for obtaining these optically pure compounds. The classical method is by using enzymatic or chemical resolution, starting from a racemic mixture. With this method, the undesired enantiomer is formed in 50%. To obtain a highly efficient process, these unwanted isomers need to be racemized and recycled.

Another method is asymmetric synthesis starting from naturally occurring compounds. For this method a stoichiometric amounts of chiral material is needed. Both the expenses and the lack of availability of optically active material are limiting factors (Petra 1999).

A more elegant way to prepare chiral alcohols is by starting from a prochiral ketone, using a catalyst. A prochiral ketone is a substrate that is not optically active because it

has a plane of symmetry, but from which the corresponding alcohol is chiral. Using asymmetric catalysts, the alcohol may be obtained in enantiomerically pure (or enriched) form since the catalyst can enter from either side of the plane. The optically active information present in the catalyst differentiates between front side and back side attack (*si*-face and *re*-face) of the substrate (figure 1.1)

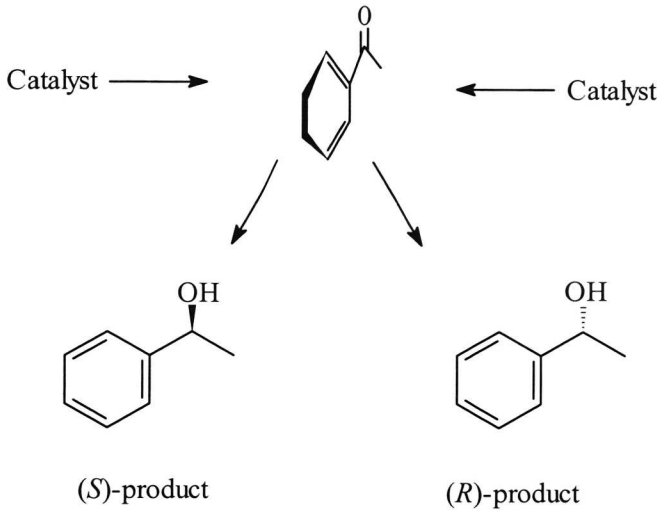


Figure 1.1 Front side and back side attack on prochiral substrate yielding optically active products

Starting from a prochiral substrate, both chemically prepared catalysts (*e.g.* for asymmetric hydrogenation or asymmetric transfer hydrogenation) and biocatalysts can be used. Excellent results have been obtained with asymmetric hydrogenation reactions, especially since the pioneering work of Noyori (1989) and coworkers. A drawback of this method, however, is the fact that often high hydrogen pressures are required. Therefore, more recently the attention has shifted towards asymmetric transfer hydrogenation reactions. The latter reductions are mostly carried out using iridium, rhodium or ruthenium catalysts in combination with a wide variety of ligands. Chiral ligands consist of a carbon backbone (in which the chirality is present) and donating atoms (Noyori and Hashiguchi 1997; Palmer and Wills 1999; Petra *et al.* 2000).

Much effort has been devoted to the development of chiral catalysts for the transfer hydrogenation of various substrates. Enantioselectivities of over 95% were obtained (Palmer and Wills 1999). A drawback of chemically prepared systems is that the presence of functional groups in ketones generally gives rise to a dramatic decrease in both the activity and selectivity of the catalyst.

As all living beings contain only single enantiomers of the constituent amino acids and sugars in their proteins, DNA and glycoproteins, biological systems contain a natural ability for production and conversion of optically pure compounds. For this reason, catalysts obtained from natural systems (biocatalysts) are useful alternatives in enantioselective reduction.

BIOCATALYTIC ASYMMETRIC REDUCTION

From an economic point of view, baker's yeast, *Saccharomyces cerevisiae*, has potential in biotransformations because it is inexpensive and easy to obtain. Of all yeasts, baker's yeast has been most intensively investigated (Reed 1983; Sybesma *et al.* 1998; Ward and Young 1990; Sato and Fujisawa 1990; Chin-Joe *et al.* 2000). Asymmetric reductions carried out by cells or enzymes of yeast have been demonstrated. Among the many types of carbonyl-containing compounds that have been reduced, the main focus has been on the successful enantioselective reductions of ß-ketoesters and ketocarboxylic acids. Structures of ß-ketoesters that possess a cyclic portion are also reduced (Ward and Young 1990). To a lesser extent, reductions of ketones and aldehydes have been investigated, along with lactones and diketonic compounds (Nakamura *et al.* 1984; Sato *et al.* 1987; Nakamura *et al.* 1989; Matsuda *et al.* 2000). Yeast cells or enzymes can catalyze a wide variety of ketones. Using crude extract of the yeast *S. cerevisiae* α,ß-unsaturated ketone trans-4-phenyl-3-buten-2-one can be successfully reduced to its corresponding alcohols with enantioselectivities up to 94% (Anorne *et al.* 1998). Acetophenone can be selectively reduced to its corresponding (R) alcohol with ee>95% using acetone powder of the yeast *Geotrichum candidum* in combination with NAD^+ as coenzyme. Various oxidoreductases of baker's yeast are surveyed in an attempt to rationalize biotransformation reactions observed in whole cell systems in terms of specific yeast enzymes. The enzymes of the greatest potential in enantiomeric reductive biotransformations utilize NADH and NADPH as coenzymes (Nakamura *et al.* 1989).

High productivity numbers (amount of product/dry weight of catalyst) using whole cells of baker's yeast cannot be achieved for fundamental reasons. Carbohydrates as electron donors must undergo five or six consecutive, enzyme catalyzed reactions before NADH is formed. Also acetaldehyde is formed in alcoholic fermentation and competes for the reduction equivalents. Formation of ethanol is usually the main reaction (Simon *et al.* 1985; Sybesma *et al.* 1998)

Keto-esters, aryl ketones and cyclic diketones are also selectively reduced by bacterial cells or enzymes (Tidswel 1991; Patel *et al.* 1992; Ensign *et al.* 1998). Bacteria capable of asymmetric ketone reduction include *Rhodococcus, Corynebacterium, Arthrobacter, Pseudomonas* and *Acinetobacter* species (Sih and Chen 1984). The isolated enzyme of *M. campoquemadoensis* can be used in the enantioselective reduction of a keto ester, producing a key intermediate of the anitiasthma drug Montelukast (Shaffiee *et al.* 1998). Recently, an example of an enantioselective reduction of a 3,5-dioxocarboxylate specifically at the 5-position using a NADP-dependent alcohol dehydrogenase from *Lactobacillus brevis* overexpressed in a recombinant *Escherichia coli* strain was described (Wolberg *et al.* 2000).
Besides synthesis by microbial hydrogenation, chiral compounds can also be synthesized using electromicrobial or electroenzymatic reduction (Simon *et al.* 1985). In the two latter methods, anaerobic or aerobic organisms are supplied with electrons from electrochemically reduced artificial mediators, *e.g.* methyl viologen (Günther *et al.* 1983). Reductases that do not require pyridine nucleotides and can accept electrons directly from reduced viologens are especially useful. The majority of alcohol dehydrogenases involved in the catalysis of ketone reduction is NADPH or NADH dependent (Plant and Cowan 1991; Kragl *et al.* 1996; Yang *et al.* 1996). Many cells contain methyl viologen-dependent NAD(P) reductases, a large number of which have still not been characterized (Günther *et al.* 1983).

Biocatalysts that contain an extensive (reductive) enzyme system and therefore have a considerable potential to produce aldehydes and alcohols from the corresponding acids and additionally perform enantioselective reductions are white-rot fungi.

The enzyme system of white-rot fungi

Basidiomycetes, and especially white-rot fungi produce a number of reductive enzymes. White-rot fungi are the best lignin degrading fungi. Lignin, one of the most abundant natural aromatic polymers on earth is found in all higher plants. Lignin provides wood with water impermeability, strength, and protection of cellulose against bacterial attack. It is a heterogenous, three-dimensional, hydrophobic structure that is highly resistant to biodegradation. Nevertheless, species of white-rot fungi such as *Phlebia radiata, Phanerochaete chrysosporium, Trametes versicolor, Bjerkandera adusta* and *Dichomitus squalens* (Muheim *et al.* 1990; Muheim *et al.* 1991; Rogalski *et al.* 1991; Gold and Alic 1993; Vares *et al.* 1995; Perié *et al.* 1996; Schick-Zapanta and Tien 1997;), are able to completely mineralize this highly resistant polymer to carbon dioxide, gaining access to, and growing on wood polysaccharides, cellulose and hemicellulose, which are the actual energy and carbon sources (Kirk *et al.* 1976). For this ligninolyses, white-rot fungi have an extensive non-specific enzyme system, composed of extracellular oxidative and intracellular reductive enzymes. Also various low molecular weight factors (a.o. veratryl alcohol, chlorinated anisyl metabolites, organic acids, maganese) play a role in the ligninolytic enzyme system of white-rot fungi.

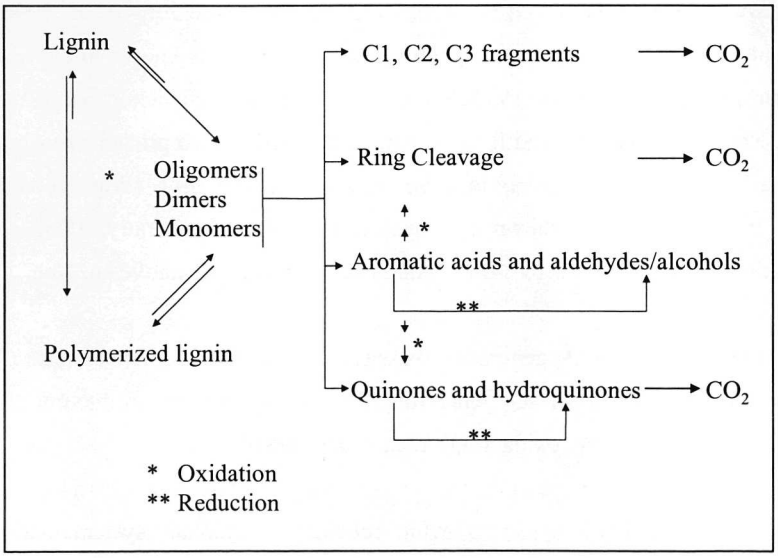


Figure 1.2 Schematic presentation of the relationship of oxidative and reductive processes leading to lignin biodegradation by white-rot fungi

By a combination of oxidation of phenolic and non-phenolic compounds and reductions of some aromatic acids, aldehydes and ketones, all aromatic rings in the lignin polymer are either converted to ring opened products or to quinones, which can be further reduced to hydroquinones. These products are then further metabolized to carbon dioxide (figure 1.2) (Schoemaker 1990).

The oxidative part of the enzyme system is the most intensively studied. This extracellular ligninolytic system responsible for the oxidation of lignin and related aromatic compounds is composed of a very complex enzymatic machinery. Well known are Lignin Peroxidase (LiP), found in several white rot species (Harvey *et al.* 1985; de Jong *et al.* 1994), and Manganese Peroxidase (MnP). Both peroxidases require H_2O_2 for activity. The catalytic cycle is like those of other peroxidases. However, LiP is a remarkable enzyme in the sense that it is capable of oxidizing electron-rich, non-phenolic aromatic compounds at low pH values (Kersten *et al.* 1990; Schoemaker 1990). The native enzyme is activated in the presence of H_2O_2 to form compound I, a two-electron oxidized intermediate. Compound I returns to the native state via compound II by two separate one-electron oxidations of aromatic substrates. An appropriate substrate that can be oxidized by compound II is required for closing the catalytic cycle. Veratryl alcohol (3,4-dimethoxybenzyl alcohol), a secondary metabolite produced by many white-rot fungi (de Jong *et al.* 1994), is a good substrate of the compound II intermediate of LiP and is known to complete the catalytic cycle (Harvey *et al.* 1989; Koduri and Tien 1995; Schick-Zapanta and Tien 1997). Reduction of compound II to the native state of LiP also prevents the oxidation of compound II to the relatively inactive compound III by H_2O_2 (Wariishi and Gold 1989). In addition it was shown that veratryl alcohol or the veratryl alcohol cation radicals can convert the inactive LiP compound III back to the native enzyme.

H_2O_2 is produced by H_2O_2 generating oxidases such as aryl alcohol oxidase (AAO). Some white-rot fungi lack H_2O_2 generating oxidases and are, for production of H_2O_2, dependent on organic acid oxidation (Urzua *et al.* 1998).

Although the ligninolytic system of white-rot fungi has already systematically been studied since the sixties, only recently the importance of reductive conversions in the lignin biodegradation process has been emphasized (Ander *et al.* 1980; Leisola *et al.*

1988; Schoemaker and Leisola 1990; Muheim *et al.* 1991). Furthermore it was discovered that in the degradation of xenobiotics, especially chlorophenolics, the reductive system also plays an important role (Valli and Gold 1991).

Reductases of the ligninolytic system

QUINONE REDUCTASES

Quinones are formed during the mineralization of lignin and pollutants (Haemmerli *et al.* 1986; Hammel *et al.* 1986; Hammel and Tardone 1988; Mileski *et al.* 1988; Valli and Gold 1991; Valli *et al.* 1992; Joshi and Gold 1993; Higuchi 1996). They are common products formed during the oxidation of chemicals by the fungal peroxidases. Quinone formation has also been reported to occur from phenolic compounds such as vanillyl alcohol (Ander *et al.* 1980). Quinones must undergo reductions, methylations and oxidations before ring cleavage occurs and they are mineralized. Quinone degradation and mineralization was shown using radiolabeled quinones (Leisola *et al.* 1988).

One role of quinone reductases is the reduction of quinones to hydroquinones making these compounds susceptible to attack by the fungal peroxidases so they can be further involved with the lignin degrading system. Furthermore, efficient uptake and mineralization of quinones and ring-openend products are instrumental in avoiding repolymerization of fragments. Therefore, rapid reduction of quinones followed by further catabolism is one possible mechanism to shift the polymerization-depolymerization equilibrium, as shown in figure 1.2, towards degradation with formation of CO_2 (Schoemaker 1990; Schoemaker *et al.* 1990).

Both intracellular and extracellular mechanisms exist for the reduction of quinones. The intracellular quinone reductases are constitutive enzymes that use NADH or NADPH to reduce the quinones. They have been discovered in studies on the metabolic pathway of vanillic acid. The intracellular reductases have also been suggested to be involved in the degradation of veratryl alcohol (Leisola *et al.* 1988). The presence of at least two different enzyme forms acting on different quinones has been proposed (Schoemaker *et al.* 1989).

One intracellular NAD(P)H:quinone oxidoreductase has been purified from *P. chrysosporium*. It contains several isozymes with broad substrate specificity. The enzyme is induced by vanillic acid or 2-methoxy-1,4-benzoquinone (Constam *et al.* 1991; Samejima and Eriksson 1992).

The cellobiose:quinone oxidoreductase is an extracellular quinone reductase, induced by cellobiose. The enzyme is often linked with lignin degradation. It was suggested that the reduction of phenoxy radicals formed during lignin degradation may be important in preventing repolymerisation reactions (Hendriksson *et al.* 1991; Bao *et al.* 1993). Recently it was shown that cellobiose:quinone oxidoreductase is a breakdown product of cellobiose oxidase (Wood and Wood 1992).

Another mechanism that exists for the reduction of quinones is a plasmamembrane redox system. These systems can use intracellular pools of NADH or NADPH as the reductant to catalyze the reduction of chemicals in the extracellular media. The plasmamembrane redox system is much less specific since, besides quinones, it reduces 2,4,6-trinitrotoluene (TNT), tetrazolium dyes, ferricyanide, nitroaromatic compounds and certain radicals (Stahl and Aust 1993; Stahl *et al.* 1995). The plasma membrane redox system may also play an important role in the detoxification of radicals. The radicals formed by the fungal peroxidases (Popp *et al.* 1990; Shah *et al.* 1992), including oxygen radicals, are generated in close proximity to fungal hyphae and may damage the fungi by causing lipid peroxidation of the fungal membrane, leading to the death of the mycelia. Reduction of the radicals by the plasma membrane redox system can protect the fungus from oxidative damage of the fungal membrane (Stahl *et al.* 1995).

Hydroquinone-reductase activity has been shown for the white-rot fungus *Phanerochaete chrysosporium* (figure 1.3). Hydroquinones are selectively reduced to hydroxy substituted cyclohexenones, compounds that cannot readily be obtained by pure chemical means (Tuor *et al.* 1993).

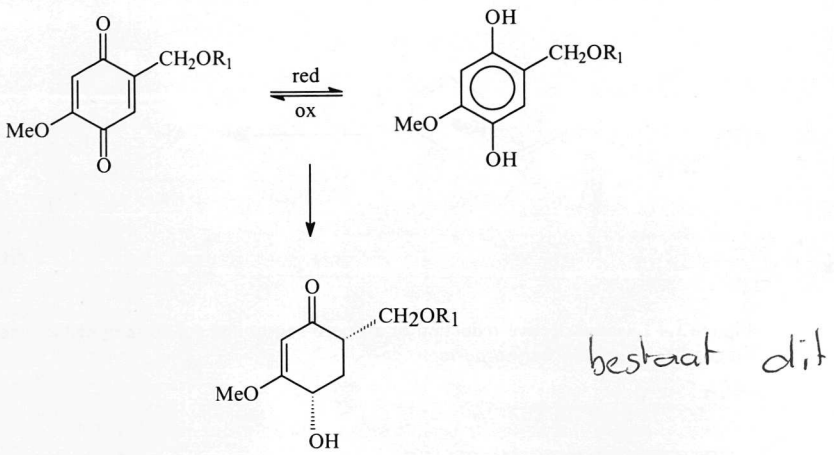


Figure 1.3 Reduction-oxidation equilibrium between quinones, hydroquinones and cyclohexonones

Other enzymes involved in further degradation of quinones and hydroquinones are demeth(ox)ylases, methyltransferases, and ring-cleaving enzymes, presumably dioxygenases (Tuor *et al.* 1993).

KETONE REDUCTASES

Little is known about ketone reduction by white-rot fungi. The reduction of the ketone 2-hydroxy-1-(4'-methoxyphenyl)-1-oxoethane to the corresponding diol has been described for cultures of *Phanerochaete chrysosporium* (Enoki and Gold 1982). The enantioselectivity of this arylsubstituted ketone reductase is not known. Nevertheless, *P. chrysosporium* contains a very selective ketone reductase, capable of a reduction of (*Z*)-3-fluoro-4-phenyl-1-(p-tolylsulphonyl)but-3-en-2-one, yielding the corresponding (*R*) alcohol in enantiomerically pure form (95% ee) (figure 1.4). Remarkably, the yeast *Geotrichum candidum* reduces the same compound, to form exactly the opposite enantiomer (98% ee) (Bernardi *et al.* 1990).

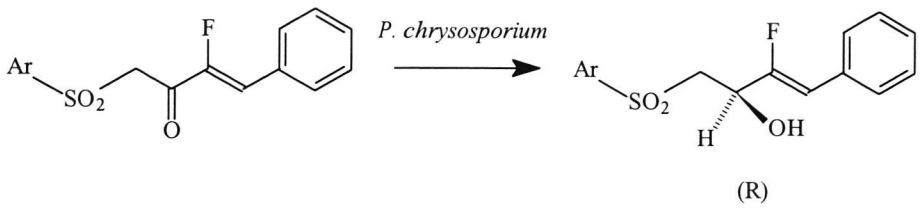


Figure 1.4 Enantioselective reduction of a fluoro-substituted α,β-unsaturated ketone by the white-rot fungus *Phanerochaete chrysosporium*

REDUCTIVE DEHALOGENASES

Reductive dechlorination reactions have recently been found in several white-rot fungi. Reductive dechlorination of chlorinated hydroquinones occurs during a multistep degradation of 2,4,6-trichlorophenol (2,4,6-TCP), a wood preservative and pesticide.

The degradation is initiated by a LiP- or MnP-catalyzed oxidation, yielding a 2,6-dichloro-1,4-benzoquinone. The benzoquinone is reduced to the corresponding hydroquinone. Reductive dechlorination occurs subsequent to quinone reduction, forming a 2-chloro-1,4-dihydroxybenene, which can be further dechlorinated to the 1,4-hydroxyquinone (Reddy *et al.* 1998).

AROMATIC ACID AND ALDEHYDE REDUCTASES

A wide range of aromatic compounds (acids, aldehydes and alcohols) with a methoxygroup at the *para*-position is synthesized *de novo* by white-rot fungi, depending on the strain, growth substrates and growth conditions (Gallois *et al.* 1990). It has been anticipated that these kind of compounds are produced via the shikimate pathway (Turner and Aldridge 1983). Veratric acid, veratraldehyde and veratryl alcohol were shown to be synthesized *de novo* from glucose in many white-rot fungi (Lundquist and Kirk 1978; de Jong *et al.* 1994). These secondary metabolites originate from *de novo* biosynthesis via L-phenylalanine.

The present aryl (veratryl, anisyl and chlorinated anisyl) acids and aldehydes are reduced to the corresponding aldehydes and alcohols by aromatic acid and aldehyde reductases. Also during lignin degradation, aromatic acids and aldehydes are released and these acids and aldehydes are reduced intracellularly by white-rot fungi as well.

Furthermore, aromatic acid and aldehyde reduction of dimeric lignin model compounds has been described for ligninolytic cultures of *Phanerochaete chrysosporium* (Schoemaker 1990). It has been known for some time that aromatic acid and aldehyde reductase (AAD) is present in whole cultures of a.o. *Trametes versicolor, Sporotrichum pulverulentum, Phlebia radiata and Phanerochaete chrysosporium.*
An important question is why basidiomycetes produce these aryl-alcohol dehydrogenases.

A plausible role of AAD during lignin degradation, is reducing aromatic acids and aldehydes to convert them into more amenable substrates for ligninolytic enzymes. Thereby continued degradation is enabled (Schoemaker *et al.* 1989; Reddy *et al.* 1998; Schoemaker 1990).

The *de novo* produced aryl (veratryl, anisyl and chlorinated anisyl) aldehydes and alcohols can serve as substrates of extracellular aryl oxidase, generating H_2O_2 for ligninolytic peroxidases (de Jong *et al.* 1994; Field *et al.* 1995). The aryl alcohol metabolites are stable in the aggressive extracellular ligninolytic system. The only significant conversion is into their corresponding aldehydes and acids. The aryl aldehydes and acids formed are intracellularly readily reduced back by NADPH dependent aryl-alcohol dehydrogenase to the alcohols, which generates a physiological cycle (figure 1.5). Presumably another role of reductive enzymes in white-rot fungi is to maintain the redox cycle of the ligninolytic system. Thus constant alcohol levels are maintained for physiological purposes without the need for energy consuming biosynthesis of additional alcohols (de Jong *et al.* 1994).

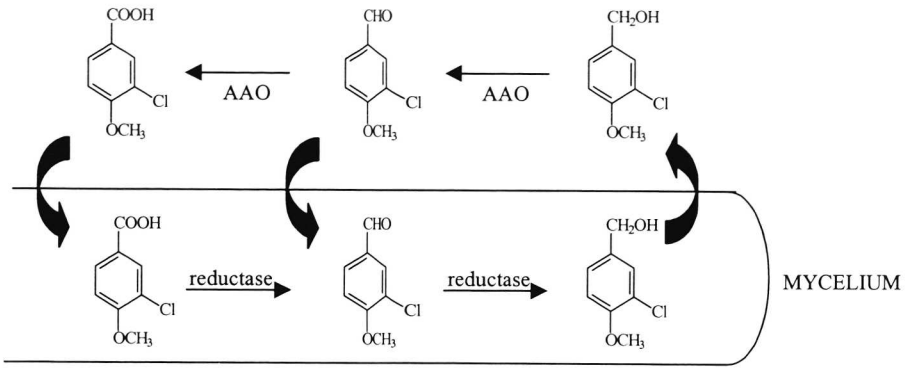


Figure 1.5 Proposed physiological redox cycle for (chlorinated) anisyl metabolites

Chlorinated anisyl metabolites such as 3-chloro-anisyl alcohol (3-chloro-4-methoxybenzylalcohol) and 3,5-dichloro-anisyl alcohol (3,5-dichloro-4-methoxybenzyl alcohol) are not just accidentally produced metabolites. Apart from their antibiotic properties (Pfefferle *et al.* 1990), they also play an important role in the ligninolytic system. Almost all fungi that produce chloroaromatics (CAM) also produce AAO (de Jong *et al.* 1994; Pelaez *et al.* 1995). CAM alcohols are much better substrates for AAO compared to the nonhalogenated metabolites veratryl and anisyl alcohol, since they have much lower k_m values for AAO. Furthermore they are much better protected against decay due to the electron-withdrawing chloro group which increases the oxidation potential of the methoxy benzyl ring (de Jong *et al.* 1994).

Other chlorinated metabolites identified from white-rot strains are the *p*-anisylpropanoid metabolites *erythro*-1-(3',5'-dichloro-4'-methoxyphenyl)-1,2-propanediol and 1-(3'-chloro-4'-methoxyphenyl)-3-hydroxy-1-propanone from *Bjerkandera* sp BOS55 and *Bjerkandera fumosa* species (Swarts *et al.* 1996). The *erythro*-1-(3'-chloro-4'-methoxyphenyl)-1,2-propanediol (trametol) is *de novo* synthesized by *Bjerkandera* sp BOS55 and *Trametes meyenii* (Ohta *et al.* 1986; Brambilla *et al.* 1995; Swarts *et al.* 1996). Trametol can be synthesized by treating fermenting baker's yeast with 3 chloro-4-methoxybenzaldehyde (which is a *de novo* produced metabolite by white-rot fungi). Probably the diol arises from reduction of α-ketols formed from aldehydes and a C_2 unit, so called C_2 homologation (*Brambilla et al.* 1995). It is not known whether the *p*-anisylpropanoid metabolites synthesized by

white-rot strains are directly produced from corresponding diketones or by C_2 homologation.

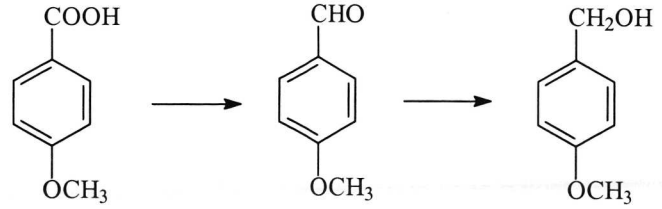
Purification of acid reductases from in white-rot fungi has never been described. Reduction of veratric acid to vertraldehyde and alcohol by crude extract has been shown for *Polystictus versicolor* (Zenk *et al.* 1965). An intracellular NADPH-dependent aryl-alcohol dehydrogenase (AAD) from the white-rot fungus *Phanerochaete chrysosporium* was purified and characterized (Muheim *et al.* 1991). This enzyme is produced during secondary metabolism and presents a broad substrate specificity, completely reducing aromatic aldehydes such as veratryl aldehyde, *p*-anisaldehyde, vanillin and 3,5-dimethoxybenzaldehyde. Ketones are not part of the substrate spectrum of this reductase. Acid reduction by this aldehyde dehydrogenase has not been described.

The use of white-rot fungi as a biocatalyst for the production of fine chemicals such as aldehydes and chiral alcohols has not been launched before. Nevertheless, as described in the above sections, white-rot fungi are able to both reduce acids to aldehydes and reduce ketones to the corresponding alcohols. Also stereoselective reduction has been reported. In addition, whole cell cultures of white rot fungi are capable of degrading a wide variety of xenobiotics, making them potentially highly robust biocatalytic systems (Schoemaker *et al.* 1991). Therefore the enzyme system of white-rot fungi might have oustanding reductive enzymes for biocatalytic applications.

Project targets and industrial and social relevance

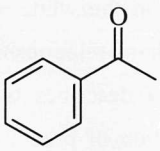
20% to 30% of the Gross National Product of the Netherlands is generated by the chemical industry. Since catalytic conversions are involved in over 80% of the total chemical production, it may be of no surprise that catalysis is of strategic interest for the Dutch industry. In order to maintain and to improve the competitive position of the Ducth industries, the Dutch Ministry of Economic Affairs promotes innovative research in a number of promising fields by funding. In this way, also the collaboration between universities, research institutes and industry is strengthened.

The research described in this thesis was financed by the Dutch Ministry of Economic Affairs via the Innovation Oriented research Programme (IOP) directed towards Catalysis. The central theme of this extensive research programme is "precision in chemical conversions." This precision is required both to save energy and feedstocks and to avoid the production of waste. Classical procedures in the manufacture of fine chemicals often involve low selectivities, the use of undesirable, toxic or corrosive reagents, and the formation of side products and large amounts of waste, causing a burden to the environment. To minimise this waste stream and to improve upon chemical yields and stereoselectivity, alternatives are necessary. Therefore, it has been decided to direct the efforts of the IOP catalysis program in particular to the development of novel catalytic routes in the fine chemical industries. Biocatalysts are well known for their high degree of selectivity and can offer a favourable alternative if they can be cheaply, and environmental friendly produced and applied on a large scale. Drawback of biocatalytic redox systems is that often expensive cofactors or co-substrates are required. This problem can be avoided by using whole cell systems. The objective of the project described in this thesis is the development of novel biocatalytic routes to aryl aldehydes, aryl alcohols and enantiopure secondary alcohols for the fine chemical industries. Acid reduction and enantioselective ketone reductions are attractive methods for the production of respectively aryl aldehydes and alcohols Whole cell systems of white-rot fungi are potentially suitabe catalysts since they have a very extensive enzyme system and are capable of a broad spectrum of conversions. Model reaction for acid reduction is the reduction of *p*-anisaat (**1a**), a *de novo* synthesized metabolite of white rot fungi, to its corresponding *p*-anisaldehyde (**1b**) and *p*-anisylalcohol (**1c**).



1a **1b** **1c**

Model substrates for ketone reduction are acetophenone (**2**), 1-(3'-chloro-4'-methoxyphenyl)-1-propanone (**3**) and 1-(3',5'-dichloro-4'-methoxyphenyl)-1-propanone (**4**). Acetophenone is often used as model substrate in catalytic ketone reductions. Furthermore it was found to be a side product in the metabolic pathway that leads from L-phenylalanine to the major aryl metabolites produced by *Bjerkandera adusta* (Lapadatescu *et al.* 2000). The substrates **3** and **4** are natural like compounds to white-rot fungi (Swarts *et al.* 1998).



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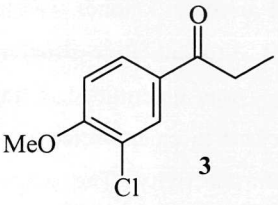

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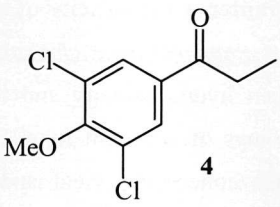

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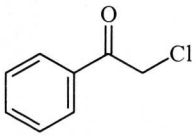
Whereas compounds 3 and 4 can be considered as model compounds for aryl-substituted substrates, the aryl ketones such as substrate **5** and **6** can be considered as models for side chain functionalisation. Moreover the corresponding alcohols are of high interest as targets for pharmaceutical applications. The product alcohol of 2-chloroacetophenone (**5**) can be converted into chiral epoxides. The corresponding chiral alcohol of 3-chloropropiophenone (**6**) results in a precursor for the homochiral form of fluoxetine, an anti-depressant.



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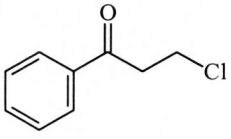

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Outline of this thesis

The objective of this PhD study was to explore the reductive enzyme system of white-rot fungi with a special emphasis on its biocatalytic properties in acid reduction and enantioselective ketone reduction.

In **Chapter 2** ligninolytic basidiomycetes were screened for their ability to catalyze the reduction of a variety of aryl acids to produce the corresponding aldehydes and alcohols. Of all strains tested, the fungus *Bjerkandera BOS55* performed best in acid reductions. The substrate spectrum of this fungus was determined.

In **Chapter 3** the toxicity of several aromatic ketones was tested on the white-rot fungus *Phanerochaete chrysosporium*. A quantitative structure-toxicity relationship between hydrophobicity and toxicity was determined. **Chapter 4** describes two screenings of white-rot fungi with regard to enantioselective reduction of prochiral ketones concerning yield and enantioselectivity. The white-rot fungus *Merulius tremellosus* ono991 performed the best. Moreover, this biocatalytic system was compared to ruthenium(II)-amino alcohol and iridium(I)-aminosulfide catalyzed asymmetric transfer hydrogenation of a broad spectrum of ketones. Physiological aspects of the biocatalytic system are described in **Chapter 5**. Incubation conditions were optimized to increase yield. Most of the yield increase was due to N_2-flushing and the attributing factors were investigated. Furthermore, ketone reduction in cell extract of *M. tremellosus* is presented.