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Summary

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

Reductions of carboxylic acids are of interest to industry since the formed aldehydes and/or alcohols have potential applications as starting materials for pharmaceuticals and as food and flavor compounds.

Reductions of carboxylic acids to their corresponding aldehydes or alcohols are energetically difficult reactions. Direct chemical reduction of carboxylic acids only became possible since 1946. Nevertheless, numerous micro-organisms are capable of acid reduction. However, a drawback of using these organisms as catalysts in acid reduction is that most systems reduce the formed aldehydes further to the alcohol, whereas in some cases the aldehydes are more valuable than the alcohols.

Chiral alcohols also form an important class of intermediates for the synthesis of fine chemicals and pharmaceuticals. In recent years, regulations on the marketing of new drugs have become more strict with respect to chiral compounds. Furthermore, most of the established routes for obtaining these optically pure compounds are inefficient and/or environmentally unfriendly. Therefore, new and efficient production methods are required. As biological systems contain a natural ability for production and conversion of optically pure compounds, catalysts obtained from natural systems (biocatalysts) are useful alternatives in enantioselective reduction.

White-rot fungi are lignin degrading basidiomycetes. The extensive ligninolytic system contains both oxidative and reductive enzymes. Although the extracellular oxidative system has been studied extensively, relatively little is known about the reductive part of the enzyme system.

This thesis describes the investigation of the reductive part of this ligninolytic enzyme system and the potential application of white-rot fungi as biocatalysts in acid reductions and enantioselective ketone reduction.

The potential use of white-rot fungi in carboxylic acid reduction was explored in **chapter 2**. Fifty-two basidiomycete strains were screened for their tolerance towards high concentrations of acids. *Dichomitus squalenes*, *Bjerkandera* sp. strain BOS55, *Phanerochaete chrysosporium*, *Trametes hirsuta*, *Phlebia brevispora* and *Schizophyllum commune* proved to be the most tolerant. An evaluation of these strains for their ability to reduce *p*-anisic acids showed that the white-rot fungus *Bjerkandera* sp. strain BOS55 was one of the best reducing strains and was highly tolerant towards high concentrations of different aromatic acids. Whereas most fungi had the tendency to reduce the acid to predominantly the alcohol, *Bjerkandera* sp. strain BOS55 appeared to have the equilibrium on the side of the aldehyde.

p-anisic, 3-chloro-4-methoxybenzoic, 3,5-dichloro-4-methoxybenzoic, 3,4-dichlorobenzoic, 4-fluorobenzoic and 3-nitrobenzoic acids were all part of the substrate spectrum. According to these results we concluded that several white-rot fungi have very good biocatalytic potential for the reduction of aryl acids.

Since aryl ketones and their corresponding chiral alcohols can be toxic towards microorganisms, the effects of these compounds on white-rot fungi were tested, prior to the screening for biocatalytic properties in ketone reduction. In **chapter 3**, tests were conducted on four aryl ketones and their corresponding reduced derivatives. Since ketone reduction by the white-rot fungus *Phanerochaete chrysosporium* had been reported before, this fungus was used as model organism. The tested ketones, acetophenone, hydroxy-acetophenone, 2-chloroacetophenone and 3-chloropropiophenone, all showed higher toxicity towards the fungus than their corresponding alcohols. A direct correlation between the hydrophobicity of the compounds and their toxicity was observed. For the model compound acetophenone, 50% growth inhibition was already shown at a concentration of 3.3 mM of the ketone.

Therefore a screening of different basidiomycete strains was performed in **chapter 4**. The aryl ketones acetophenone, 1-(3'-chloro-4'-methoxyphenyl)-1-propanone and 1-(3',5'-dichloro-4'-methoxyphenyl)-1-propanone (natural-like substrates of white-rot fungi) were reduced by all fungi. The white-rot fungus *Merulius tremellosus* reduced the ketones with the highest activity. Consequently six different *Merulius* strains were screened for both activity and enantioselectivity. The fungus *Merulius tremellosus* ono991 was used in further experiments and tested for the enantioselective reduction

of various α -aryl, dialkyl, α,β -unsaturated and chloro-substituted functionalized ketones. *M. tremellosus* ono991 appeared to have a broad substrate spectrum of prochiral ketones. The α -arylketones are reduced with enantioselectivities of 90-98%, yielding the (*S*)-configurations. Dialkyl ketones were also part of the substrate spectrum, although the desired unsaturated alcohols could not be obtained. The white-rot fungi appeared to be suitable catalysts for the enantioselective reduction of the chloro-substituted ketones. The ketones were reduced to the corresponding alcohols with enantioselectivities up to 88%. Also dehalogenation reactions were observed. In conversion studies with the α,β -unsaturated ketones, the C=C bonds were not left intact by the fungus. Remarkably, a Michael adduct was formed by the fungus by addition of H₂O to the unsaturated system.

The biocatalytic system was compared to ruthenium and iridium catalyzed asymmetric transfer hydrogenation. The two approaches appeared to be complementary. Reduction of α,β -unsaturated compounds yielded excellent results using the metal catalysts.

In **chapter 5** we optimized the culture conditions with respect to oxygen supply, choice of primary substrate and aryl ketone concentration using 1'-acetonaphthone as model compound. We demonstrated that the yield obtained in nitrogen flushed incubations with glycerol as primary substrate was increased from 57% to 98% compared to incubations under air with glucose. The enantioselectivity and product configuration remained constant. Our results showed that the yield increase due to N₂ flushing could be attributed to two factors. First an increased stability of the product α -methyl-1-naphtalenemethanol in anaerobic compared to aerobic atmosphere was demonstrated. Second fermentative metabolism increased reduced enzyme cofactors available for the reduction.

The dependency of ketone reductase for NAD(P)H was demonstrated by the reduction of 1'-acetonaphthone in cell extracts of *M. tremellosus* ono991.