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Improvement of Cytochrome-P450-Mediated Biotransformations by Filamentous Fungi

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Cytochrome P450 enzymes are found throughout nature and form a superfamily of enzymes with *ca.* 500 members identified to date. P450's are monooxygenases involved in conversion of substrates such as (poly)aromatic hydrocarbons, steroids, long-chain alkanes and fatty acids, terpenoids, *etc.*, and are often rate-limiting in a process.

P450's are characterized by the presence of a haem group covalently bound to a cysteine present in a conserved region in the C-terminal part of the protein. Where there is hardly any sequence identity between different members of this superfamily, all enzymes have a well-conserved 3-D structure.

In eukaryotic organisms, cytochrome P450 enzymes occur in two different cell compartments, mitochondria and microsomes. The majority of cytochrome P450 enzymes in eukaryotes are found anchored in the membrane of the endoplasmatic reticulum with the catalytic site protruding in the cytosol. This class of cytochrome P450 enzyme systems consists of two proteins, a reaction-specific cytochrome P450 enzyme and a general working cytochrome P450 reductase (CPR), an enzyme that transports electrons from NADPH to the cytochrome P450.

Examples of industrial biocatalytic processes in which cytochrome P450 enzymes are involved include the production of dicarboxylic acids from alkanes, the production of site-specific hydroxylated steroids and the production of hy-

droxylated aromatic molecules. Furthermore, cytochrome-P450-mediated biodegradation of PAH-contaminated waste/soil is being studied.

To make application of P450-mediated biotransformations commercially attractive, we have set out a study to improve biocatalytic activities of fungal P450 enzymes. In a previous study, we showed that overexpression of the cloned (cyto-

chrome P450) benzoate *para*-hydroxylase gene (*bphA*; *cyp53*) of the filamentous fungus *Aspergillus niger* resulted in increased mRNA and protein levels but not in improved hydroxylase activity levels or product formation [1]. From studies in yeast, it was known that overexpression of cytochrome P450 genes is most efficient in combination with overexpression of the NADPH cytochrome P450 reductase (the second component of the cytochrome P450 enzyme system) [2]. Therefore, one of the possible explanations for the absence of an increase in enzymatic activity in *bphA* multicopy strains was that NADPH cytochrome-P450 reductase activity is limiting. To verify this hypothesis, we have isolated the *cpr* gene of *A. niger* [3][4] and have tested its overexpression in cytochrome P450 multicopy strains. Therefore, multiple copies (m.c.) of the *cprA* gene were introduced into *A. niger* wild type and into *A. niger* transformants containing multiple copies of the *bphA* gene creating a set of four different strains (wild type, m.c. *bphA*, m.c. *cprA*, m.c. *bphA* + m.c. *cprA*). Probably due to the hydrophobicity and/or toxicity of the substrate, it

Table. Benzoic-Acid Consumption (HPLC) of Different Strains After Growth for 5 h in Minimal Medium + 0.1% Benzoic Acid

	Copy number <i>bphA</i>	<i>cprA</i>	Benzoic acid [mM]
Medium			6.7
Wild type	1	1	1.15
m.c. <i>cprA</i>	1	6	0.6
m.c. <i>bphA</i>	10	1	2.4
m.c. <i>bphA</i> /m.c. <i>cprA</i>	10	10	0.0056

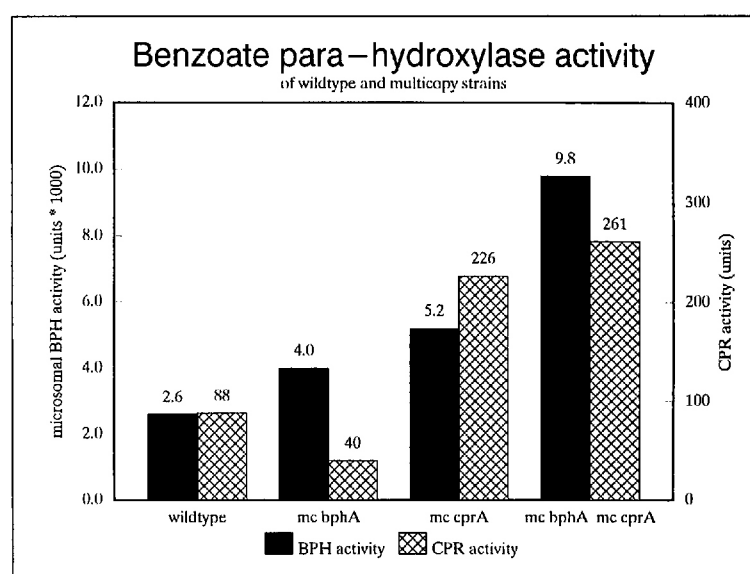


Fig. 1. Benzoate *para*-hydroxylase and cytochrome P450 reductase activities measured in microsomal fractions of *Aspergillus niger* strains with different gene copy numbers of the *bphA* and *cprA* gene (m.c.: multiple copy)

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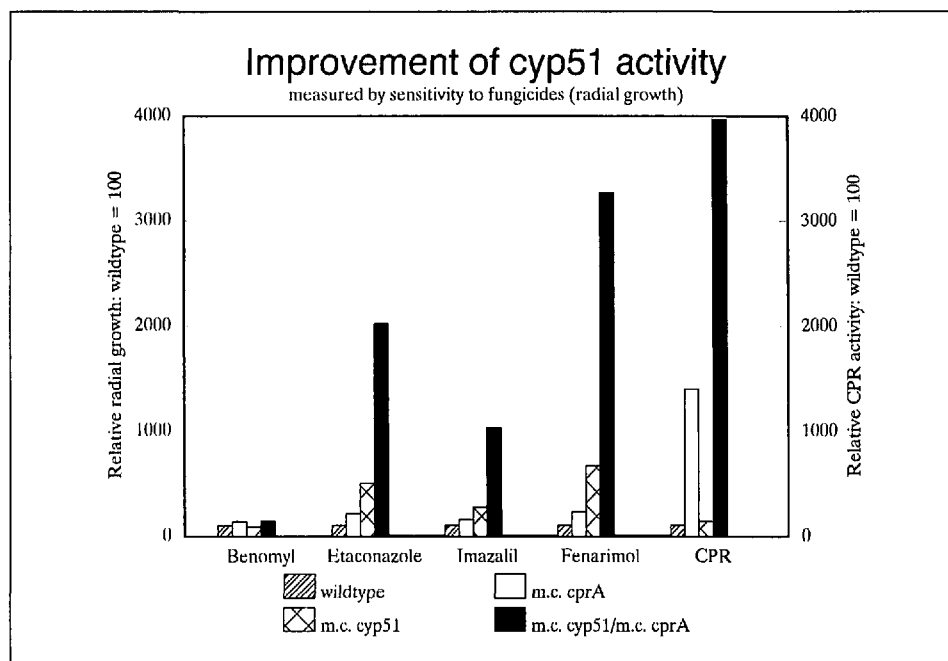


Fig. 2. Radial growth (as a measure of fungicide resistance) of test strains on plates containing different concentrations of three inhibitors of eburicol 14 α -demethylase (etaconazole, imazalil, fenarimol) and one fungicide that does not inhibit this specific enzyme (benomyl; control). For each strain also the microsomal cytochrome P450 reductase activity is shown; m.c.: multiple copy.

was very difficult to obtain reproducible *in vivo* benzoate *para*-hydroxylation (BPH) biotransformation results. A typical example of the results of such an experiment is shown in the Table. Reproducible quantification of the BPH-enzymatic activity was possible in microsomal extracts of the *A. niger* transformants. The results of these experiments are shown in Fig. 1. A small increase of BPH activity is seen

both when the *bphA* gene or the *cprA* gene was overexpressed. However, maximal activities were obtained when both genes of the enzyme system were overexpressed.

To verify these results, we also overexpressed another fungal cytochrome P450 gene. For this, we used the *Penicillium italicum* gene encoding eburicol 14 α -demethylase (*cyp51*) recently cloned by Van Nistelrooy and coworkers [5]. We

again made a set of four strains (wild type, m.c. *cyp51*, m.c. *cprA*, m.c. *cyp51* + m.c. *cprA*). Since it is difficult to directly quantify the eburicol 14 α -demethylase activity, we used in this case an indirect test with whole cells. In this test, differences in sensitivity against 14 α -demethylase inhibitors were determined in the different strains. The results of these experiments are shown in Fig. 2. A clear increase of fungicide resistance was found especially in strains in which multiple copies of both genes were present.

From both sets of experiments, it can be concluded that optimization of fungal, P450-mediated bioconversions requires overexpression of both the reaction-specific cytochrome P450 and the cytochrome P450 reductase.

- [1] R.F.M. van Gorcom, J.G. Boschloo, A. Kuyvenhoven, J. Lange, A.J. van Vark, C.J. Bos, J.A.M. van Balken, P.H. Pouwels, C.A.M.J.J. van den Hondel, *Mol. Gen. Genet.* **1990**, *223*, 192.
- [2] T. Sakaki, M. Akiyoshi-Shibata, Y. Yabasaki, K. Manabe, H. Urakami, H. Ohkawa, *Pharmacogen.* **1991**, *1*, 86.
- [3] J.M. van den Brink, R.F.M. van Gorcom, PCT Int. Pat. Application PCT/WO/94/29453.
- [4] J.M. van den Brink, C.M.J. van Zeijl, C.A.M.J.J. van den Hondel, R.F.M. van Gorcom, *DNA Cell Biol.* **1995**, *14*, 719.
- [5] J.G.M. van Nistelrooy, J.M. van den Brink, J.A.L. van Kan, R.F.M. van Gorcom, M.A. de Waard, *Mol. Gen. Genet.* **1996**, *250*, 725.

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Stereochemistry of Enzymatic Hydrolysis of Nitriles

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Nitriles constitute a unique class of compounds in organic chemistry. However, one of the drawbacks of conversion of nitriles is that they are generally resistant to hydrolysis, and hence, rather drastic reaction conditions are required. In this way, enzyme-catalyzed hydrolysis of nitriles is one of the most important biotransformations of synthetic substrates, as it can be carried out under mild reaction conditions [1]. Hydrolysis of a cyano group is achieved without affecting ester and

acetal functionalities, which is very difficult *via* ordinary chemical reactions.

It is already established that there are three kinds of enzymes which concern in hydrolysis of nitriles, as shown in Scheme 1. In most cases, reaction proceeds in two steps *via* intermediary formation of an amide. These two steps are catalyzed by two enzymes. Thus, in some cases, hydrolysis reaction can be stopped at the stage of an amide, without forming any trace of a carboxylic acid [2]. Another

interesting feature of nitrile-hydrolyzing enzymes is their stereoselectivity. Enzymes attack the positive carbon of a nitrile and an amide with recognition of the chirality or prochirality of the same carbon skeleton. Thus studies on the stereoselectivities of a nitrile hydratase and an amidase would be a good model for understanding how enzymes are controlling the stereochemical course of the reactions.

We have screened a nitrile-hydrolyzing microorganisms using benzonitrile and/or 3-hydroxypropionitrile as the sole source of nitrogen, and found that an actinomycetes, *Rhodococcus rhodochrous*, was the most active strain, which is now deposited

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