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Microbial screening in hydroxylation of L-proline

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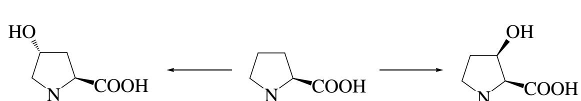
Abstract—Microbial screening of 250 wild type strains resulted in identification of five strains with the activity of prolyl hydroxylase. All five strains hydroxylated regioselectively and enantioselectively L-proline into 4(*R*)-*trans*-hydroxy-L-proline **1**. The best conversions were obtained with a wild type of *Aeromonas caviae*. 3-Hydroxylase activity was not detected.

The importance of enzymatic hydroxylations is in their ability to introduce one or more OH groups at inactivated centers in hydrocarbons. First microbial hydroxylations described in the literature were with steroids as substrates.[1–4] Hydroxylations of hydrocarbons other than steroids have been extensively studied with $P450_{cam}$ from *Pseudomonas putida*, which transforms D-camphor into 5-*exo*-hydroxycamphor. Small and medium-size cycloalkenes have been hydroxylated and mixtures of allyl hydroxylated products accompanied with epoxydes and/or cis-diols have been obtained.[5,6] In the series of aromatic compounds, the hydroxylation is often the initial step in the degradation and detoxification in both procaryotes and eucaryotes yielding a mixture of phenols and catechols. Some microorganisms hydroxylate benzylic positions and other heteroaromatic side chains. Thus hydroxylation of ethyl benzene, for example, leads to enantiomerically enriched phenylethanols.[7] Hydroxyprolines, 4(*R*)-*trans*-hydroxy-L-proline (**1**) and 3(*R*)-*cis*-hydroxy-L-proline (**2**), Figure 1, are useful chiral synthons in the synthesis of pharmaceuticals. They play an important role in the preparation of anti-inflammatory pharmaceuticals like oxaceprol, inhibitors of angiotensin converting enzyme like fosinopril, or even food additives.



1 **2**

Figure 1.

The enzymes responsible for hydroxylation of L-proline are prolyl 3-hydroxylase and prolyl 4-hydroxylase, respectively. Both enzymes were described in *Streptomyces*,[8–11,13,14b] *Bacillus*,[8,12,15] *Dactylosporangium*[14a,b] and *Amycolatopsis*[14a] species and are not very abundant. The screening of more than 3000 microorganisms resulted in only eight strains able to produce prolyl hydroxylase activities. The activity of prolyl hydroxylase was very weak in some cases and thus detectable only by using C-14 labeled substrate.[10,11] *Dactylosporangium* sp. proline 4-hydroxylase gene has been cloned and expressed in *Escherichia coli*[14a] as a fused protein that had 13-fold higher activity then the enzyme in the original strain. Recombinant 4-hydroxylase from *Dactylosporangium* sp. and 3-hydroxylase from *Streptomyces* sp. have been assayed for their selectivity with different substrates.[16] Prolyl hydroxylases were partially purified and characterized. Both prolyl hydroxylases require 2-oxoglutarate as a cosubstrate and Fe^{2+} as a cofactor. EDTA, Zn^{2+}, Cu^{2+}, Co^{2+} and Ba^{2+} are reported to be the enzyme inhibitors.[10] The crystal structure of the first proline 3-hydroxylase with and without complexed iron from *Streptomyces* sp. has been elucidated.[17] There is no data on three dimensional structures of other 2-oxoglutarate oxygenases. Thus the authors suggest the possibility of the convergent evolution to a mechanism/active site chemistry with different overall folds. Among fungi, 3-hydroxylase and 4-hydroxylase have been detected in the pneumocanding-producing strain of *Glarea lozoyensis*.[18] The enzymes converted L-proline to both *trans*-3- and *trans*-4-hydroxy-L-prolines in the reaction dependent on 2-oxoglutarate, ascorbate, dithiotreitol and Fe^{2+}.

Keywords: L-Proline; Hydroxylation; Microbial screening.
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We describe in this letter a new, rapid and original screening approach. Although the described methodology was applied in the microbial screening of prolyl hydroxylase activity, it can be applied to any kind of bioconversion. Two hundred and fifty wild type strains have been screened for their ability to selectively hydroxylate L-proline. A rapid screening was set up on a small scale (96-well format, Fig. 2) with the cultures grown on the solid medium as described in the experimental part. The first and the most important reason for using solid agar plugs rather than liquid cultures in the screening is the presence of, extracellular and intracellular enzymes. Moreover, this approach allows the long term storage of biocatalysts with a minimal loss of activity. As the plugs are kept frozen in the sterile buffer of the desired pH they are ready to be used when needed.

When microorganisms grew and covered completely the surface of the medium on Petri plate they were cut and placed in the sterile buffer of defined pH depending on the reaction searched. Among 250 strains screened for prolyl hydroxylase activity, 31 strains transformed more than 30% of L-proline. However, only five microorganisms (Table 1) gave the product whose retention time, UV and mass spectra correspond to 4(*R*)-*trans*-4-hydroxy-L-proline **1**, while the remaining 26 strains metabolized L-proline without the appearance of detectable product(s). L-Proline was stable under the same reaction conditions and no decomposition or degradation was detected when the biocatalyst was omitted. Similarly, strains alone did not produce any detectable hydroxyl proline when the substrate was omitted.

The screening was realized in 1 ml reaction volume and the positive results were reproduced several times in a 24-well format with 10 ml reaction volume. The best reproducibility was obtained with *Aeromonas caviae*, and this strain was used for the flask experiments. Quantitative HPLC analysis obtained with *A. caviae* shows 68% of conversion of starting substrate. However, the 4(*R*)-*trans*-hydroxy-L-proline **1** was obtained in only 10% of yield. The product was quantified from HPLC/UV spectra after its transformation into 2,4-dinitrofluorobenzene derivative. The identification of the biotransformation product was based on comparison of its MS spectra with those of the commercial samples after the transformation into 2,4-dinitrofluorobenzene derivative. The HPLC spectra of one example of *A. caviae* biotransformation products and of the standard commercial samples are given in Figures 3 and 4. The crude lyophilized product from a culture broth of *A. caviae* was analyzed by NMR and compared to commercial hydroxyl prolines. The NMR analysis confirmed the 4(*R*)-*trans*-hydroxy-L-proline **1**.

Other microorganisms which hydroxylated L-proline into *trans*-4-hydroxyproline (*Bacillus subtilis*, *Fusarium solani* and *Gordonia rubripertincta*) did not grow better and moreover showed lower activity then *A. caviae*. In order to better exploit the enzymatic potential of *A. caviae* prolyl hydroxylase on a large scale we put

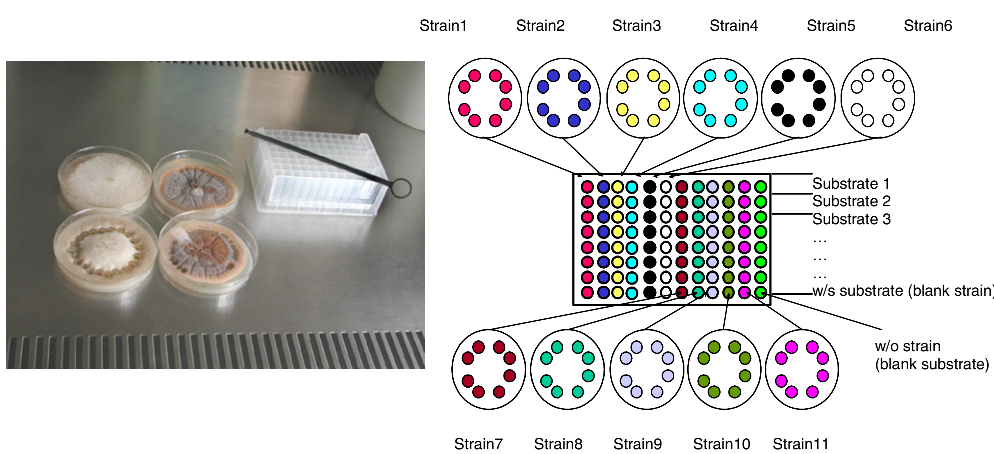

Figure 2.

Table 1. HPLC results of biotransformation of L-proline

Strain	Origin of strains	[Dry biomass] (mg/ml)	Conv.[a] (%)	4-OH-Pro (%)
Bacillus subtilis	ATCC 21967	7.00	88	8
Aeromonas caviae	CRTL	5.75	68	10
Fusarium solani	CRTL	5.80	93	6
Gordonia rubripertincta	CRTL	7.90	74	7
Staphylococcus capitis	CRTL	5.15	96	6
Blank (substrate w/o strain)[b]	—	—	0	0

[a] HPLC conversion based on L-Pro being consumed.

[b] L-Proline was stable under the same reaction conditions. No decomposition or degradation was detected when the biocatalyst was omitted.