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Preparative regio- and chemoselective functionalization of hydrocarbons catalyzed by cell free preparations of 2-hydroxybiphenyl 3-monooxygenase

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Abstract

Oxygenases are useful catalysts for the selective incorporation of molecular oxygen into hydrocarbons. Here, we report on the application of isolated, cell free 2-hydroxybiphenyl 3-monooxygenase (HbpA) as catalyst for the regio- and chemospecific hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl. The catalyst was prepared from recombinant *Escherichia coli* using expanded bed adsorption chromatography and could be stored without significant loss of activity in lyophilized form. The reaction was performed in an aerated and thermostated simple stirred glass vessel in an aqueous (20% v/v)/organic (80% v/v) reaction medium. This allowed in situ product recovery preventing substrate and product inhibition of the catalyst as well as decay of the labile product 2,3-dihydroxybiphenyl. Enzymatic regeneration of reduced nicotinamide cofactors was achieved using the formate/formate dehydrogenase system. We obtained volumetric productivities of up to 0.45 g l^{-1} h^{-1}. No significant decrease of productivity was observed within 7 h and more. Product purification (purity 92%) was achieved using solid phase extraction with aluminum oxide followed by crystallization as a polishing step (purity > 99%).

To our knowledge, these results show for the first time the perspectives of integrated enzyme and cofactor regeneration-based biocatalytic processes in organic/aqueous emulsions, coupled with in situ product recovery.

Keywords: Biocatalysis; 2-Hydroxybiphenyl 3-monooxygenase; Cell free biotransformation; Enzyme reactor; Bioprocess

1. Introduction

Bioprocesses using isolated enzymes as catalysts show significant advantages over processes based on either growing or non growing whole cells [1]. Such in vitro applications of enzymes often result in higher volumetric productivities as compared to whole cell-based processes. Also, the apparatus used and the work up of reaction products is simple. In part, this is due to the fact that the production of the biocatalyst, and hence the growth of the production organism, is uncoupled from the biotransformation. This allows separate optimization of both processes, which is a clear advantage over the application of living cells, as conditions for optimal cell growth and

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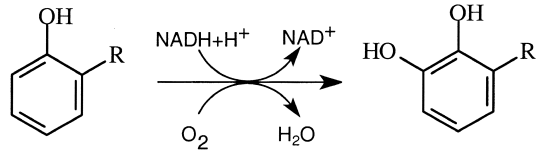
enzyme production in cells can be very different from the reaction conditions used for chemical synthesis. In order to achieve high productivities with oxygenases in whole cells, the cells have to be metabolically highly active. This assures an optimal regeneration capacity of the cells for reducing nicotinamide cofactors. This is achieved best using growing cells [1] which, in addition to their primary metabolism, turn over non natural substrates to the desired products in so called co-metabolic reactions [2].

Today, the cell free concept is mainly realized for hydrolytic enzymes, which are not dependent on stoichiometric amounts of cofactors. In contrast, oxidoreductases like dehydrogenases and oxygenases are dependent on coenzymes, for example NAD(P)H. Application of these enzymes in isolated form on a preparative scale is only economical if suitable coenzyme recycling systems are used (reviewed in Ref. [3]). One of the best and most widely used is the formate dehydrogenase/formate system, which has also been applied successfully in enzyme membrane reactors [4,5]. Enzymes used in these setups were mostly dehydrogenases, but in a few cases members of the more complex class of oxygenases were also used [6]. Enzyme membrane reactors allow the separation of expensive enzymes and coenzymes during semicontinuous and continuous biotransformations resulting in high space time yields and minimized enzyme inhibition by substrates or products. On the other hand, while allowing isolation of reaction products, the advantage of using isolated enzymes in a simple apparatus with simple handling is lost. First attempts to use oxygenases in stirred tank reactors in aqueous medium were successful on a small scale, e.g. for the Baeyer Villiger-type cyclohexanone monooxygenase together with formate dehydrogenase [7]. This two-enzyme system was used in an aqueous reaction medium, which necessitated extraction of the product in a second step after biotransformation.

In this report, we describe the use of isolated 2-hydroxybiphenyl 3-monooxygenase (HbpA) for the preparation of 2,3-dihydroxybiphenyl from 2-hydroxybiphenyl. HbpA catalyzes the first step in the degradation pathway of 2-hydroxybiphenyl in the soil bacterium *Pseudomonas azelaica* HBP1 [8]. The enzyme has been well characterized biochemically



2-Hydroxybiphenyl 3-monooxygenase

R = Ph, 2'OH-Ph, 2,3$(OH)_2$-Ph
F, Cl, Br
Me, Et, Pr, *i*-Pr, But

Fig. 1. Substrate spectrum of HbpA.

[9,10] and shows a broad substrate spectrum allowing the regio- and chemospecific hydroxylation of a range of phenols to their corresponding catechols (Fig. 1). Recombinant HbpA has been used as a biocatalyst in whole *Escherichia coli* cells for the preparation of various 3-substituted catechol derivatives [11–14].

2. Experimental

2.1. Materials

2,3-Dihydroxybiphenyl was obtained from Wako (Neuss, Germany). Solvents were from Biosolve (Zurich, Switzerland). All other chemicals used were of analytical grade and were obtained from Fluka (Buchs, CH). Formate dehydrogenase was a kind gift of Prof. Wandrey (Jülich, Germany).

2.2. Activity assays and determination of protein concentration

The activity of HbpA was determined as the 2-hydroxybiphenyl dependent rate of NADH oxidation at 25°C and in the presence of 0.2 mM 2-hydroxybiphenyl as described previously [8]. The enzymatic activity of formate dehydrogenase was determined spectrophotometrically by following the reduction of NAD^+ at 340 nm and 30°C for 1 min. The reaction mixture of 1 ml contained 160 μmol sodium formate, 1.6 μmol NAD and formate dehydrogenase in 50 mM potassium phosphate buffer pH 7.5. The specific activity of formate dehydrogenase used in this study was 2.14 U/mg. One unit (U) of enzyme activity was defined as the

amount of enzyme forming 1 μmol of product per minute.

Protein contents were measured with the Bio-Rad protein assay kit (BioRad Laboratories, Munich, Germany) based on the Bradford method with bovine serum albumin as a standard.

2.3. Enzyme preparation

Biomass of *E. coli* JM101 pHBP461 containing HbpA was prepared essentially as described earlier [14] on a 150-l scale. Cells (650-ml paste) were disrupted by two passages through a French Press (Aminco, USA) at 170 MPa. The lysate was diluted up to 3.15 l with 100 mM Tris/HCl buffer pH7.5 and incubated with 10 000 U Benzonase® for 30 min at room temperature. For recovery and purification of HbpA, the lysate was loaded on a Streamline 50 column (Pharmacia) containing 1395-ml Streamline DEAE anion exchanger (Pharmacia) at a flow rate of 100 ml min^{-1} (pump: Watson Marlow 502S). The expanded bed was washed with 1000 ml (65 ml min^{-1}) 20 mM Tris HCl pH 7.5 containing 7.5% glycerol, and 1800 ml (100 ml min^{-1}) 20 mM Tris HCl pH 7.5. HbpA was eluted from the compressed bed with 20 mM Tris HCl pH 7.5 using a linear gradient of NaCl from 0 to 1 M NaCl formed by a chromatography system (Labomatic Instruments, Allischwil, CH) at 10°C with detection of protein at 280 nm and collection of fractions (approx. 55 ml each). HbpA was recovered in four pooled fractions at 150 to 200 mM NaCl. The different pooled fractions had specific activities of 0.57 U/mg (570 ml), 0.48 U/mg (385 ml), 0.14 U/mg (325 ml), and 0.09 U/mg (330 ml) resulting in a recovery of a total of 8984 U and a loss of 71 U by washing the expanded gel bed as described above. This corresponds to a total recovery of 2.57-g HbpA with an overall purity of about 10% with respect to total cell protein. HbpA of this quality could be stored at −20°C for several months without loss of activity. Results described in this report were obtained with an HbpA fraction having a specific activity of 0.48 U/mg and a purity of 14% with respect to total cell protein.

2.4. Lyophilization

HbpA was lyophylized for easy handling and storage by freezing samples of 1 to 30 ml in liquid nitrogen and drying for 16 h in a Lyovac GT3 (Leibold Heraeus). HbpA was lyophilized directly in pooled fractions collected after expanded bed chromatography of crude extracts of *E. coli* JM101 pHBP461.

2.5. Biotransformation

Biotransformations were performed in a 250-ml glass reactor under non-sterile conditions. The reactor was stirred with a magnetic bar (250 rpm) at 30°C. The total reaction volume was 200 ml consisting of 160 ml decanol and 40 ml 50 mM potassium phosphate buffer pH 7.5. Reaction components are listed in Table 1. Oxygen was supplied directly to the reaction medium in saturating amounts.

The amount of 2,3-dihydroxybiphenyl in the organic phase was analyzed every 15 min: samples of 250 μl were taken from the suspension, acidified with 4 μl of 10% perchloric acid, and centrifuged for 10 s at $15\,800 \times g$ (Eppendorf 5415) to obtain phase separation. The organic phase was analyzed using straight phase HPLC. The HPLC system (Merck Hitachi) consisted of a L-5000 LC controller, 655A-40 autosampler, 655A variable wavelength UV monitor and 655A-R liquid chromatography pump and was fitted with a CC 250/3 Nucleosil 100-5 CN column (Macherey-Nagel). Samples were eluted under isocratic conditions using 96% hexane, 4% isopropanol and 0.1% H_2O pH 2 at a flow rate of 0.66 ml min^{-1} resulting in a pressure of 64 to 67 bar. The elution pattern was monitored at 210 nm (retention times were 2.51 min for 2-hydroxybiphenyl and 6.68 min for 2,3-dihydroxybiphenyl). The concentration of 2,3-dihydroxybiphenyl in the aqueous phase was about 0.16% of the concentration detected in the organic phase.

2.6. Product recovery and polishing

At the end of the reaction, the two phases were separated by centrifugation at 5000 rpm and 4°C for 3 min (Sorval SS 34 rotor; Sorvall RC-513 centrifuge, Du Pont Instruments). The organic phase was applied to a column filled with 60 g of neutral aluminum oxide C507. The product was eluted from the column using methanol: 0.5 M HCl (9:1). The

Table 1
Setup of cell free hydroxylation of 2-hydroxybiphenyl

	Composition of reaction medium	
	Aqueous phase	Organic phase
Solvent	50 mM potassium phosphate pH 7.5 (40 ml)	decanol (160 ml)
Components	160 mM sodium formate 22.5 U HbpA[a] 33.75 U formate dehydrogenase 0.2 mM NADH 20 μM FAD	110 mM 2-hydroxybiphenyl

[a]Cell free HbpA (HbpA, 0.48 U/mg, 14% of total cell protein) was prepared as described in Section 2.

elution pattern was followed by HPLC analysis. Fractions containing product (> 92% pure as determined by HPLC) were pooled (650 ml) and the liquor was reduced to dryness. The crude product was digested in about 1% w/v hexane (60°C) and left at room temperature for crystallization of 2,3-dihydroxybiphenyl [14]. Crystals were recovered using 0.2-μm filters (Ligacon), washed with cold water and finally dried.

3. Results and discussion

3.1. Preparation of the enzymes

HbpA was recovered and enriched directly from disrupted cells without clearing of the crude extract by expanded bed adsorption chromatography as described above. In addition to the removal of non-protein impurities this unit operation resulted in an enrichment factor of 1.3.

We developed a lyophylisation protocol to achieve simple handling and storage of HbpA. HbpA in pooled fractions from chromatography was lyophilized directly, diluted fivefold in 50 mM Tris/HCl pH 7.5 (buffer A) and diluted five times in 50 mM potassium phosphate buffer pH 7.5 (buffer B). Dry samples were rehydrated with H_2O and specific activities were determined as follows: 0.2 U/mg (not diluted), 0.31 U/mg (diluted in buffer A), and 0.42 U/mg (diluted in buffer B). The corresponding relative activities of lyophilized HbpA with respect to not lyphilized enzyme were: 42%, 64%, and 87%, respectively. HbpA was therefore lyophilized in buffer B for storage and shipping. This should be done starting preferably from desalted HbpA containing solutions as 10 mM NaCl already inhibits HbpA activity by 30% [9].

Efficient protocols for the production of formate dehydrogenase including handling of this enzyme are reported [15].

3.2. Stability of HbpA and formate dehydrogenase in the presence of organic solvents

Oxygenases are complex enzymes. The overall reaction of the organic substrate with molecular oxygen and reduced nicotinamide cofactors is frequently subject to substrate and/or product inhibition, especially for apolar substrate/product pairs. Using such oxygenases, biotransformations yielding high product concentrations at high volumetric productivities are only feasible with immediate in situ recovery of products from the reaction medium during the reaction. This is true for both whole cell- and cell free-applications of oxygenases. Held et al. [14] described a whole cell process for recombinant HbpA in growing cells of *E. coli* with in situ recovery of 2,3-dihydroxybiphenyl on XAD4 as a solid phase. The application of a second, organic liquid phase for the in situ product recovery in whole cell processes using organic/aqueous suspensions is also well reported in literature [16–18].

Isolated dehydrogenases for oxidoreduction of steroids including enzymatic coenzyme recycling have also been applied in such two-liquid phase systems consisting of an aqueous phase and an organic phase [19]. Methods for the recycling of cofactors in organic reaction media have been reviewed by Adlercreutz [20]. In aqueous organic suspensions enzymes get in direct contact with organic solvents. We therefore tested the specific activities of formate

dehydrogenase and HbpA in the presence of 50% organic solvents (Fig. 2). Formate dehydrogenase showed 100% activity in 50% hexane ($\log P$ 3.5), whereas HbpA showed 100% activity in 50% decanol ($\log P$ 4). For formate dehydrogenase high specific activities from 75% to 100% of its maximal activity in buffer were determined for solvents with $\log P$ values between 2 and 8.8. This reflects a relatively high stability of formate dehydrogenase in a broad range of organic solvents. When comparing the influence of different organic solvents on HbpA, we observed a narrow tolerance of HbpA towards organic solvents. The highest specific activity for HbpA was determined in 50% decanol. Thus, HbpA activity seems to be reduced in very apolar organic solvents. Based on these results, biotransformations employing both HbpA and formate dehydrogenase were performed in decanol/buffer suspensions.

3.3. Cell free biotransformation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl

The reaction principle used for the biotransformation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl using isolated HbpA and formate dehydrogenase is shown in Fig. 3. Decanol was used as a reservoir of educts and a sink for products via in situ extraction in a simple stirred tank reactor in a suspension with buffer (composition see Table 1). This setup allowed the use of high concentrations of 2-hydroxybiphenyl, which would have been prohibitive in an aqueous reaction medium due to substrate inhibition of HbpA [9]. In the two-liquid phase system, product decay due to autooxidation as well as product inhibition of HbpA were circumvented because the product is extracted from the aqueous phase directly after its formation. Partition coefficients for 2-hydroxybiphenyl and for 2,3-dihydroxybiphenyl were determined to be 1700 and 650, respectively. The resulting low concentration of 2-hydroxybiphenyl in the aqueous phase did not negatively influence the reaction rate under the conditions tested, since the K_m value of HbpA for 2-hydroxybiphenyl is 2.8 μM [9].

Fig. 4 shows the formation of 2,3-dihydroxybiphenyl from 2-hydroxybiphenyl in a reaction setup as described in Table 1. The overall activity of the two enzyme system consisting of HbpA and formate dehydrogenase was stable for at least 7 h and resulted in a productivity of 0.45 g l^{-1} h^{-1} (Table 2). No other byproducts were detected in the reaction

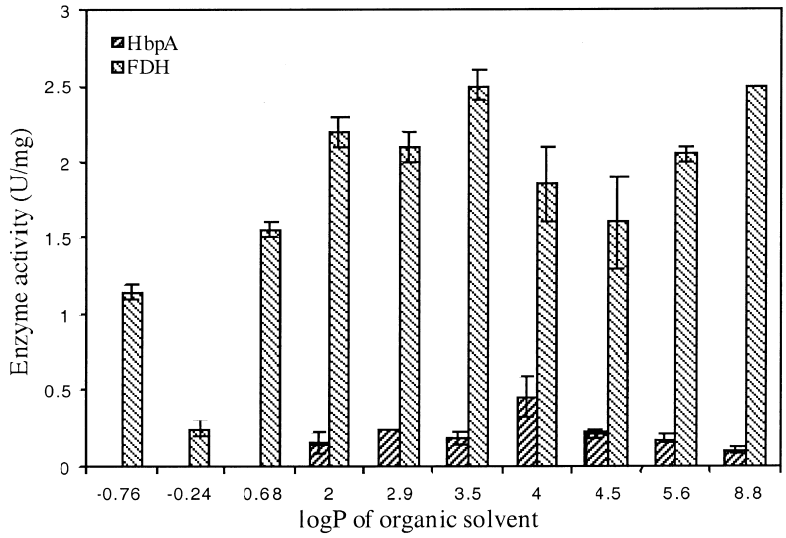


Fig. 2. Effect of $\log P$ on the stability of HbpA and formate dehydrogenase (FDH) in the presence of 50% organic solvent. Enzyme activities were determined spectrophotometrically in the aqueous phase after rigorous mixing. In case of formation of two phases, the organic phase was removed prior to determination of enzyme activity. Log P values (17): −0.76 = methanol, −0.24 = ethanol, 0.68 = ethylacetate, 2 = chloroform; 2.9 = octanol; 3.5 = hexane; 4 = decanol; 4.5 = octane; 5.6 = decane; 8.8 = hexadecane.

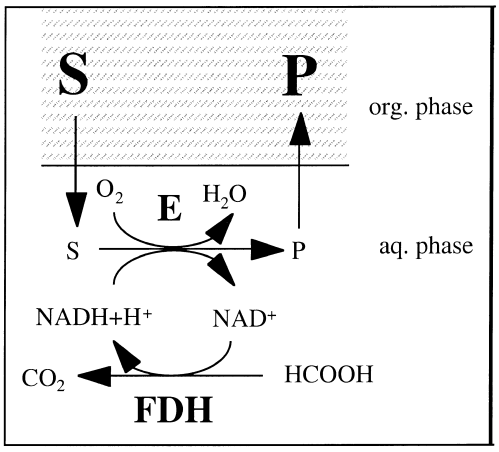


Fig. 3. Reaction principle. Application of oxygenases (E) in organic/aqueous emulsions with regeneration of the cofactor NADH from NAD by formate dehydrogenase (FDH). Substrate (S) is continuously supplied via the organic phase which acts as a reservoir and at the same time as a sink for product (P) which is extracted in situ.

mixture. Using an optimized whole cell process based on recombinant HbpA in *E. coli* for the preparation of 2,3-dihydroxybiphenyl from 2-hydroxybiphenyl, Held et al. [14] reported a productivity of 0.39 g l^{-1} h^{-1} over 10 h. This shows the competitiveness of applying isolated HbpA as catalyst in a two-liquid phase system.

Table 2
Results of cell free hydroxylation of 2-hydroxybiphenyl

Volumetric productivity	0.45 g l^{-1} h^{-1}
Yield (based on formation of 2,3-dihydroxybiphenyl)[a]	14.6%
Turnover number of NADH (based on product formation)	403
Activity of HbpA over reaction time	0.079 U mg^{-1}

[a]No by products were formed. Total turnover of 2-hydroxybiphenyl was 15%.

A turnover number for NADH of 403 was calculated based on the amount of 2,3-dihydroxybiphenyl formed. However, the actual turnover number might be higher because of the NADH oxidase activity of HbpA. This so called pseudosubstrate activity of HbpA is explained by the reduction of oxygen to hydrogen peroxide with electrons derived from NADH in a reaction running in parallel to its oxygenase activity [9]. The specific rate of 2,3-dihydroxybiphenyl formation over the reaction time was 0.079 U/mg. This points to a limitation of the overall reaction rate of the two enzyme system. One reason could be the inhibition of HbpA by formate. In

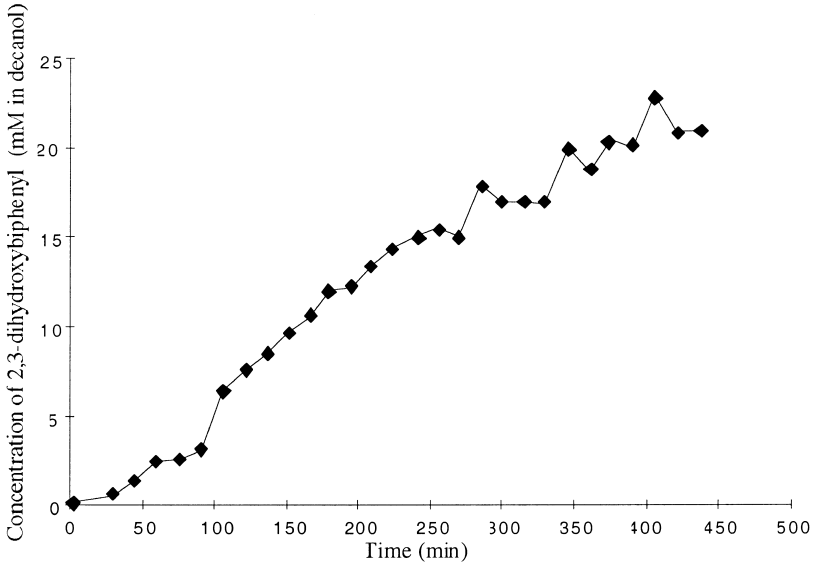


Fig. 4. Accumulation of 2,3-dihydroxybiphenyl in the organic phase over time. The setup of the biotransformation is described in Section 2 and in Table 1.

Table 3
Testing the selective adsorption of 2,3-dihydroxybiphenyl on aluminum oxide[a]

Step	Compound[b]	
	2-Hydroxybiphenyl (mg)	2,3-Dihydroxybiphenyl (mg)
Sample loaded on column[c]	6 (100%)	125 (100%)
wash 1[d]	5.5 (92%)	26 (21%)
wash 2[e]	–	–
elution[f]	–	93 (74%)

[a]Adsorbent: 5 g aluminum oxide 507C (pH 7 ± 0.5) filled in a glass column.
[b]Quantification was done by reversed phase HPLC analysis as described previously [14].
[c]Both compounds were dissolved in 25 ml 90% methanol 10% M9 minimal salts medium 10 mM HCl [21].
[d]Washing step 1: 10 ml H_2O.
[e]Washing step 2: 10 ml H_2O followed by 10 ml methanol and 20 ml methanol pH 2 (10 mM HCl).
[f]Elution: 30 ml 90% methanol 10% 5 M HCl.

spectrophotometric assays, HbpA activity decreased by 29% in the presence of 160 mM formate. This effect could partially be compensated by the addition of 10 μM FAD (decrease of 8% activity of HbpA).

A further increase of productivity might be achieved by increasing the concentration of HbpA and/or the application of a higher-enriched preparation of HbpA. As a consequence, this might increase the yield of this biotransformation, an effect that would also be expected using longer reaction times.

3.4. Product recovery and purification

2,3-Dihydroxybiphenyl was recovered from the organic phase by adsorption to aluminum oxide. Neutral aluminum oxide selectively binds 2,3-dihydroxybiphenyl with a capacity of about 20 mg 2,3-dihydroxybiphenyl per gram aluminum oxide under the conditions tested (Table 3). 2-Hydroxybiphenyl was retained very poorly by the matrix and could be washed off completely without elution of bound 2,3-dihydroxybiphenyl. Application of this method resulted in a recovery of 2,3-dihydroxybiphenyl from the decanol phase with a purity of 92% as determined by HPLC. Recrystallisation gave 2,3-dihydroxybiphenyl with a purity of > 99% (HPLC) and a yield of 95% with respect to the total amount of 2,3-dihydroxybiphenyl formed in the process.

4. Conclusions

The results presented here show that isolated HbpA is a suitable catalyst for organic synthesis, which can be handled as an 'off the shelf reagent' for biotransformations in aqueous organic/reaction media. In addition, formate dehydrogenase could be used for enzymatic regeneration of reduced NAD in these emulsions. High productivities of such processes are dependent on the optimal selection of the organic solvent with respect to stability of enzyme activities and the partition coefficients of educts and products. The productivity for the formation of 2,3-dihydroxybiphenyl as obtained in this study is comparable to that reported for an optimized whole cell catalyst based on HbpA [14].

This suggests the feasibility and shows the perspectives of the concept applying an isolated oxygenase with enzymatic cofactor regeneration as catalyst in a two-liquid phase biotransformation.

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