

Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis

Wouter A Duetz*, Jan B van Beilen and Bernard Witholt

The unique catalytic properties of oxygenases (the regio-specific and/or enantio-specific hydroxylation of non-activated carbons) are of undisputed biosynthetic value. Factors that govern the economics of their industrial use include a low k_{cat} , a frequently decreased k_{cat} in recombinant strains, limiting oxygen transfer rates in bioreactors, product inhibition, and the demanding discovery (screening) process.

Addresses

Institute of Biotechnology, ETH Hönggerberg, HPT, CH 8093, Zürich, Switzerland

*e-mail: duetz@biotech.biol.ethz.ch

Current Opinion in Biotechnology 2001, 12:419–425

0958-1669/01/\$ – see front matter

© 2001 Elsevier Science Ltd. All rights reserved.

Abbreviation

PCR polymerase chain reaction

Introduction

The past decade has seen tremendous progress in the use of enzymes, not only in food production and detergents but also in the production of fine chemicals. Of most note in this respect is the use of lipases and proteases for the production of chiral building blocks by enantio-selective hydrolysis (resolution) of racemic mixtures of esters [1]. Many organic chemists have been willing to use (partially) purified enzyme preparations, as they are as easy to handle as ‘normal’ heterogeneous catalysts and generally show higher (enantio)-, chemo- and regio-selectivity. In some cases, however, the removal of enzymes from their natural environment (i.e. the microbial cell) offers no advantages or, worse, has adverse consequences for the enzyme. This is especially true for oxygenases. There are four main reasons to use whole cells rather than purified enzymes.

First, if the presence of other enzymes has no negative impact on the purity of the product, the use of whole (dead or live) cells is simply more economical; enzyme isolation and purification are significant cost-factors. In addition, the ‘packaging’ of enzymes in small ‘bags’ of membranes and cell walls (as nature does) protects the enzyme from shear forces, and might result in a longer half-life of enzyme activity in stirred bioreactors.

Second, the membrane-bound nature of the target enzyme may be a reason to use whole cells. Removal of an enzyme from a membrane environment often leads to full or nearly complete loss of activity. Alkane hydroxylase is a well-known example of such an integral membrane protein.

Third, cascades of enzymatic reactions (e.g. in the biosynthesis of secondary metabolites like penicillin or cephalosporin) may be too complicated to perform *in vitro*

because of the number of enzymes, cofactors and substrates that are involved.

Fourth, the stoichiometric consumption of NAD(P)H, NAD(P)⁺, ATP or other cofactors during the enzymatic reaction or chain of reactions may make the use of whole cells attractive. Important NAD(P)H-dependent processes include hydroxylations/epoxidations and the reduction of ketones to chiral alcohols.

In this review, we discuss the potential and limitations of hydroxylations and epoxidations. In addition, a small number of recently developed/discovered biocatalysts is discussed. For a more detailed review on enzymatic hydroxylation reactions, we refer to a recent article of Holland and Weber [2**]

Hydroxylations and epoxidations: promises and constraints

Chemical oxidative processes that involve the introduction of oxygen atoms are often non-specific and/or depend stoichiometrically on toxic and expensive reagents (e.g. selenium dioxide or 3-chloroperbenzoic acid). The potential of enzymes in this area has been recognized for decades and the number of biocatalysts available for oxidative bioprocesses is growing steadily ([2**] for examples see Table 1). Biochemically, epoxidations and hydroxylations are very similar and many oxygenases catalyze both reactions; however, the number of these processes established in industry is still quite limited. Below, we highlight a number of factors that limit the industrial implementation of whole-cell hydroxylations and epoxidations, and discuss how and to what extent these limitations can be alleviated.

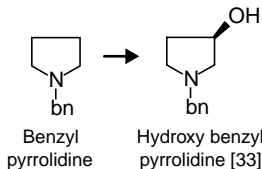
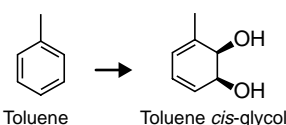
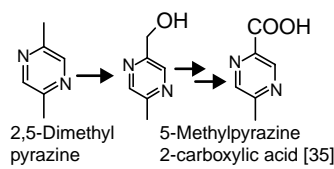
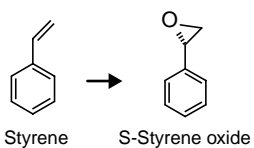
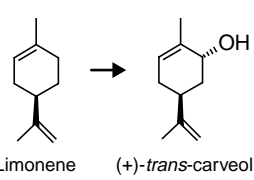
Limitation 1: turnover numbers of oxygenases

Most oxygenases are dependent on NADH or NADPH. NAD(P)H-dependent bacterial oxygenases generally show k_{cat} values in the range 0.2 to 75 s⁻¹ (Table 2). There is a widespread perception that cytochrome P450 enzymes have relatively low k_{cat} values; however, Table 2 shows that the k_{cat} values of bacterial P450 hydroxylases, like camphor-5-monooxygenase ($k_{\text{cat}} = 20 \text{ s}^{-1}$) and BM-3 from *Bacillus megaterium* ($k_{\text{cat}} = 26\text{--}75 \text{ s}^{-1}$), can be higher than those of bacterial non-heme iron hydroxylases that act on non-activated carbons, like the toluene and alkane monooxygenases ($k_{\text{cat}} = 0.5\text{--}20 \text{ s}^{-1}$; Table 2).

The underlying reasons for the relatively low k_{cat} values of NAD(P)H-dependent bacterial oxygenases (in comparison with hydrolases, for example, which often have a k_{cat} exceeding 100,000 s⁻¹) are not well known, and may be different for various oxygenases. It has been suggested (e.g. [3]) that the

Table 1

Examples of hydroxylases from bacterial catabolic pathways applicable for the preparation of fine-chemicals using whole cells.

Enzyme system	Organism	Activity (U[g dry wt] ⁻¹) (substrate)*	Reference	Example of an industrially relevant reaction [†]
Alkane monooxygenase (A)	<i>Pseudomonas putida</i> GPo1 <i>E. coli</i>	30 (Nonene) 30 (Nonene)	[4] [4]	 Benzyl pyrrolidine → Hydroxy benzyl pyrrolidine [33]
Toluene dioxygenase (B)	<i>Pseudomonas putida</i> F1 <i>E. coli</i>	750 (Toluene) 235 (Toluene)	Unpublished data [34]	 Toluene → Toluene cis-glycol
Xylene monooxygenase (C)	<i>Pseudomonas putida</i> mt-2 <i>E. coli</i>	185 (Toluene) 100 (Styrene)	[6] [5]	 2,5-Dimethyl pyrazine → 5-Methylpyrazine 2-carboxylic acid [35]
Styrene monooxygenase (D)	<i>Pseudomonas putida</i> S12 (blocked mutant) <i>E. coli</i> (<i>styA</i> , <i>styB</i>)	200 (Styrene) 90–180 (Styrene)	[7] [8]	 Styrene → S-Styrene oxide
Toluene dioxygenase (E)	<i>Rhodococcus opacus</i> PWD4	15 (D-Limonene)	[36]	 D-Limonene → (+)-trans-carveol

*If available, specific activities for the original host strain and for *E. coli* recombinants are given. [†]Reference included if different from previous column.

electron transfer step of oxygenase reactions is a relatively slow step. The observation that monooxygenases and dioxygenases that do not involve NAD(P)H show a 10-fold–1000-fold higher k_{cat} value (Table 2) provides some support for this assumption. Protocatechuate-3,4-dioxygenase ($k_{cat} = 758 \text{ s}^{-1}$), gentisate-1,2-dioxygenase ($k_{cat} = 642 \text{ s}^{-1}$) and nitropropane dioxygenase ($k_{cat} = 6400 \text{ s}^{-1}$) are the most illustrative examples in this respect (Table 2).

The NAD(P)H-dependent oxygenases with the highest k_{cat} values ($5\text{--}75 \text{ s}^{-1}$; Table 2) enable theoretical specific activities of intact cells of $150\text{--}4500 \text{ U (g dry wt)}^{-1}$ (where 1 U is defined as 1 μmol of product formed per minute), assuming that the size of the oxygenase is 50 kDa per active site and represents 5–10% (w/w) of total cell protein. The lower end of this range of theoretically possible whole-cell activities has been reached in several instances; for example, the dihydroxylation of toluene by toluene dioxygenase expressed in *Escherichia coli*, the epoxidation of styrene, and the hydroxylation of the methyl group of

toluene by xylene monooxygenase (Table 1). Activities significantly higher than $500 \text{ U (g dry wt)}^{-1}$ have so far not been reached in practice — this may be for several reasons.

Lower k_{cat} values in recombinant strains

Heterologous expression in a foreign host may lead to a lower k_{cat} so that more enzyme needs to be produced in the recombinant to obtain the same specific whole-cell activity as in the wild-type strain. This has been found to be the case for alkane monooxygenase from *Pseudomonas putida* GPo1 [4^{*}]. Expression of this alkane monooxygenase in *E. coli* W3110 resulted in a maximal specific activity of $30 \text{ U (g dry wt)}^{-1}$, which is identical to the activity in the wild-type strain although the oxygenase content was six-fold higher in the recombinant. Another example is provided by xylene monooxygenase (also an integral membrane protein); until now, its overexpression in *E. coli* has not resulted in enhanced specific activities (maximally $\sim 100 \text{ U [g dry wt]}^{-1}$ [5] in comparison with the optimally induced wild-type strain *P. putida* mt-2 $185 \text{ U [g dry wt]}^{-1}$

[6]). Also, if one compares the activity of styrene monooxygenase in *P. putida* S12, in which the enzyme was originally discovered (200 U [g dry wt]⁻¹ [7]), with the activity of the best *E. coli* construct carrying the genes encoding a similar styrene monooxygenase (90–180 U [g dry wt]⁻¹ [8]), it is clear that heterologous overexpression does not necessarily lead to higher activities. This may be because of the different membrane properties of the artificial host (such as the absence or presence of cyclopropane groups or mycolic acids; length, degree of saturation, and conformation [*cis-trans*] of fatty acid moieties of phospholipids), instability of the electron-transfer component (e.g. as found for naphthalene dioxygenase [9]), the requirement for a reactivating component (as found for catechol-2,3-dioxygenase [10]) or a shorter protein half-life (as found for the alkane monooxygenase system [4*,11]). High-level expression in the natural host ('self-cloning') may be a suitable solution for most of these problems but, surprisingly, there are no examples of this approach.

Regeneration rates of NAD(P)H

For oxygenases that stoichiometrically consume NADH or NADPH, the rate of their regeneration may limit the apparent k_{cat} above a certain expression level. Assuming that the reduction equivalents are derived from glucose (consumed in cells growing with a yield of 50% at a rate of maximally 2 g cells (g glucose)⁻¹ h⁻¹), the rate of NADH regeneration is approximately 720 U [g dry wt]⁻¹ if ammonium is used as a nitrogen source and the elemental ratio of biomass is assumed to be CH_{1.67}N_{0.2}O_{0.27}. There are no major reasons to assume that the potential maximal activity is significantly lower in cells used for the biotransformation, in which the NADH is channelled mainly to the oxygenase rather than to the electron-transfer chain. Therefore, although limitations may occasionally occur under suboptimal conditions, natural NADH regeneration rates may not be expected to limit oxygenase activities in the order of 100 U (g dry wt)⁻¹. However, if the higher end of the theoretical oxygenase activity range (1000–4500 U [g dry wt]⁻¹) were to come within reach, NADH regeneration rates may become limiting. This issue might be addressed by overexpression of enzymes involved in the generation of reduction equivalents from glucose or another source of reduction equivalents.

Limitation 2: oxygen-transfer rates in reactor systems

For many oxygenases, the K_m for oxygen is in the order of 10–60 μM [12], which is one or two orders of magnitude higher than that of the electron-transfer chain (~1 μM [13]). As a result, oxygen-limited conditions may lead to a drastic decrease in space-time yields because the oxygenase cannot compete successfully for the available oxygen. To obtain 80% saturation of an oxygenase with a typical K_m of 30 μM, oxygen must be maintained in excess of 100 μM. In practice, this requirement calls for a careful adaptation of the bacterial cell density to its specific oxygen demand (endogenous respiration plus oxygenase activity) and the oxygen-transfer rates in the bioreactor used. Large-scale bioreactors (>10 m³) have mass-transfer coefficient (k_{La}) values in the order of

Table 2

k_{cat} values of oxygenases.

Enzyme	Substrate	k_{cat} (s ⁻¹)	Ref.
Heme iron/NAD(P)H-consuming			
BM-3	Pentadecanoic acid	75	[37]
	Arachidonate	53	[38]
	Laureate	26	[39]
Camphor-5-monooxygenase	Camphor	20	[40]
	Camphor	18	[41]
Narbomycin hydroxylase	Narbomycin	1.4	[42]
Dihydrodaunorubicin oxygenase	Dihydrodaunorubicin	0.6	[43]
CYP2C18	2-Arylthioophene	2.1	[44]
CYP2C19	Mephenytoin	0.3	[45]
CYP2F2	2-Methylnaphthalene	1.1	[46]
CYP2C9	Diclofenac	0.5	[47]
CYP2E1	Ethanol	0.3	[48]
Non-heme iron/NAD(P)H-consuming/acting on non-activated carbons			
Phenylalanine 4-monooxygenase	Phenylalanine	14	[49]
Toluene dioxygenase	Toluene	9.4	[50]
Methane monooxygenase	Butane	8.8	[51]
Biphenyl dioxygenase	Biphenyl	7.3	[52]
Alkane monooxygenase	Nonene	3.8	[4]
	Octane	3.9	[53]
Toluene 4-monooxygenase	Toluene	2.0	[54]
Naphthalene dioxygenase	Naphthalene	1.8	[55]
Toluene 2-monooxygenase	Toluene	0.5	[56]
Benzoate 4-monooxygenase	Benzoate	0.2	[57]
Non-heme iron/NAD(P)H-consuming/acting on activated carbons			
3-Hydroxybenzoate 4-monooxygenase	3-Hydroxybenzoate	29	[58]
4-Hydroxybenzoate 1-monooxygenase	4-Hydroxybenzoate	20	[59]
Cyclohexanone monooxygenase	Cyclohexanone	17	[60]
Phenol 2-monooxygenase	Phenol	13	[61]
Hydroxybiphenylhydroxylase	Hydroxybiphenyl	6.2	[53]
Cyclopentanone monooxygenase	Cyclopentanone	4.2	[62]
Non-heme iron/no NAD(P)H consumption			
2-Nitropropane dioxygenase	2-Nitropropane	6400	[63]
	2-Nitropropane	1224	[64]
Protocatechuate-3,4-dioxygenase	Protocatechuate	758	[65]
Gentisate-1,2-dioxygenase	Gentisate	642	[66]
Catechol-2,3-oxygenase	Catechol	187	[67]
1-Hydroxy-2-naphthoate dioxygenase	1-Hydroxy-2-naphthoate	114	[68]
L-Lactate monooxygenase	L-Lactate	104	[69]
6-Chlorohydroxyquinol 1,2-dioxygenase	6-Chlorohydroxyquinol	72	[70]

The k_{cat} values have been categorized according to the absence or presence of heme moieties and their dependence on reduction equivalents (e.g. NAD(P)H).

200 h⁻¹ [14]. In such a bioreactor, an average air pressure of 2.5 atm and a desired residual oxygen concentration of 100 μM (corresponding to ~0.5 atm air pressure) results in an oxygen-transfer rate of 90 mmol L⁻¹ h⁻¹. This oxygen-transfer rate corresponds to 1500 U L⁻¹ which allows a maximal cell density of 10 g dry wt L⁻¹ at a specific oxygen consumption rate of 150 U (g dry wt)⁻¹ (assuming an endogenous respiration of 100 U [g dry wt]⁻¹ and an average oxygenase activity of 50 U [g dry wt]⁻¹). These figures correspond to an upper limit of the space-time yield of 30 mol m⁻³ h⁻¹ or 4.5 kg m⁻³ h⁻¹ (108 kg m⁻³ day⁻¹) if the product has a molecular weight of 150 Da. From these calculations, we also conclude that space-time yields of whole-cell hydroxylations and epoxidations using cells with activities above 100 U (g dry wt)⁻¹ become primarily constrained by the bioreactor configuration (rather than by the biocatalyst). Another implication is that at relatively low specific activities (e.g. ~10 U [g dry wt]⁻¹) the space-time yields cannot simply be enhanced by increasing the cell density: above a certain cell density endogenous respiration will lead to oxygen limitation. In such cases (if specific activities cannot be increased by mutagenesis or genetic engineering), it could be useful to develop special hosts in which the flux of reduction equivalents (e.g. from glucose) to the terminal electron chain is minimized, leading to reduced endogenous respiration and thus to the possibility of using higher cell densities. Technical solutions to increase oxygen-transfer rates (and thus the maximally attainable space-time yields) include the use of oxygen-enriched air and/or an increased pressure. Also, the presence of a second dispersed organic phase has been shown to be beneficial for oxygen-transfer rates [15].

Limitation 3: product inhibition

Space-time yields in the order of 30 mol m⁻³ h⁻¹ (as calculated in the previous section to be the upper limit in large bioreactors with k_{La} values of 200 h⁻¹) are only relevant if they can be maintained for a significant period of time. For many processes yielding alcohols or epoxides, product inhibition, which already occurs in the low millimolar range, would start to have a negative impact within 10 min. One approach to handle this problem is to continuously remove the product *in situ*. If inhibition is exerted at the enzyme level, mutant enzymes that are not inhibited can often be generated. In other cases, especially with lipophilic alcohols and epoxides (e.g. styrene epoxide), the product is toxic to the whole cell and causes disruption of the cell membrane [16]. The use of host cells with a higher solvent resistance may partially alleviate this problem and allow higher product concentrations to accumulate. For example, *cis-trans* isomerases [17] and efflux systems [18*,19] may supply a host strain with an increased tolerance to membrane-disruptive products. A more promising approach is the use of a two-phase fermentation systems that allows the *in situ* extraction of lipophilic products, thus keeping the aqueous concentration of the product at non-toxic levels [20].

Discovery of new enzymes

The discovery of new oxygenases requires screening methods that are essentially different from those used for

enzymes such as hydrolases. Whereas hundreds of hydrolytic enzymes (e.g. lipases and proteases) are commercially available, only a few oxygenases can be purchased on the market: a number of P450 enzymes (Gentest Inc., Human Biologics International, Oxford Biomedical Research Inc., PanVera Inc., Research Diagnostics Inc., Sigma-Aldrich, Xenometrics Inc., XenoTech llc) primarily used to predict the metabolic fate of pharmaceuticals, small amounts of a few Baeyer–Villiger monooxygenases, and hydroxybiphenyl monooxygenase. Furthermore, expression cloning techniques (i.e. the selection of clones from gene expression libraries prepared from DNA isolated from environmental samples or individual strains, for example, used by Diversa Inc.), which have proven to be a strong tool in the discovery and improvement of hydrolases and other uncomplicated enzymes, are generally inefficient for oxygenases. This is possibly because the genes encoding the various subunits of oxygenases may not be clustered and/or expressed well in hosts like *E. coli*. Therefore, the traditionally laborious screening of large collections of wild-type strains is often the best option to discover a desired oxygenase activity. The recent development of a system for the parallel handling of large collections of wild-type strains on microtiter plates has eased and speeded up this task significantly [21*,22] and resulted in the discovery of a number of new hydroxylation reactions (Table 1; A and D). New enzymes belonging to oxygenase classes of which at least a few have been cloned and sequenced can also be obtained using the polymerase chain reaction (PCR) with highly degenerate primers on template DNA, which is likely to yield novel oxygenases [23*]. PCR typically yields internal gene fragments which can be used as probes to clone complete genes, as used for alkane hydroxylases [24], or combined with the 5'- and 3'-ends of a well-characterized member of the same gene family, as for catechol-2,3-dioxygenases [25].

What are the main sources of industrially relevant oxygenases? The presently available microorganisms useful in biohydroxylations can be roughly divided into two classes. The first class comprises bacterial strains with hydroxylases involved in catabolic pathways. In some cases an intermediate in the degradation of the natural substrate is the desired product (e.g. toluene *cis*-glycol or styrene epoxide; Table 1 reactions B and D). Because the desired compounds do not usually accumulate, blocked mutants of the natural strain or genetic constructs in other strains are required. In other cases, catabolic hydroxylases appear to show a desired (co-metabolic) activity on unnatural substrates (examples A, C and E; Table 1). In these cases, the hydroxylated product is usually not further converted, enabling the screening of wild-type strains. The second class of microorganisms that are useful in biohydroxylation includes fungi from genera like *Cunninghamella*, *Beauveria* and *Aspergillus*. These fungi produce P450 enzymes with a broad substrate range and in general show much lower whole-cell activities than the bacterial strains expressing catabolic enzymes. The rapid increase in knowledge regarding the heterologous expression of P450 enzymes is likely to boost the discovery and use

Table 3

Estimation of the minimum costs of large-scale whole-cell hydroxylations.

Biocatalyst	Process	Fermentor costs (\$ [kg product] ⁻¹)	Medium costs (\$ [kg product] ⁻¹)	Total (\$ [kg product] ⁻¹)
A 50 U (g dry wt) ⁻¹ 10 g dry wt l ⁻¹	Fed-batch, cell growth 12 h bioconversion 12 h	0.93	0.46	1.39
B 50 U (g dry wt) ⁻¹ 10 g dry wt l ⁻¹	Continuous culture (D = 0.05 h ⁻¹), cell growth and bioconversion phase combined	0.46	0.28	0.74
C 75 U (g dry wt) ⁻¹ 10 g dry wt l ⁻¹ Strain with low rate of endogenous respiration	Continuous culture (D = 0.05 h ⁻¹), cell growth and bioconversion phase combined	0.31	0.19	0.50
D 250 U (g dry wt) ⁻¹ 4.5 g dry wt l ⁻¹ Strain with low rate of endogenous respiration	Continuous culture (D = 0.05 h ⁻¹), cell growth and bioconversion phase combined	0.21	0.06	0.27

Minimum costs of whole-cell hydroxylations per kilogram of product in standard 200 m³ stirred-tank bioreactors with estimated running costs of 50 \$ m⁻³ day⁻¹ and a specific oxygen-transfer rate of 90 mmol l⁻¹ h⁻¹,

for the production of a compound with a molecular weight of 150 kDa. Media costs for generation of cell mass are assumed to be 2.5 \$ (kg dry wt)⁻¹. Product recovery costs are not included.

of such enzyme systems for new biocatalytic processes. Recent examples in this respect are the cloning of P450 enzymes from *Cunninghamella elegans* [26] and *Aspergillus parasiticus* [27]. The number of potential sources for new P450 enzymes is massive, especially as there is little reason to exclude eukaryotic P450 enzymes from potential biocatalytic applications. A literature search yielded recent articles on the cloning of P450 enzymes from organisms as diverse as cockroaches, eels, silkworms, hamsters, chickens, and a wide range of plants. The SWISSPROT database contains almost 800 sequences of P450 enzymes (May 2001). Nowadays, the cloning of P450 enzymes is often possible by PCR using degenerate primers [28]. New sequences of whole genomes invariably result in the discovery of new genes encoding P450 enzymes. An interesting example is the large number (at least 20) of P450 sequences in the genome of *Mycobacterium tuberculosis* [29]. A recent article of Sakaki and Inouye [30] reviews the potential practical applications of human cytochromes P450. The potential for protein engineering P450 enzymes in order to increase the activity and/or specificity for unnatural substrates was recently reviewed by Miles *et al.* [31].

Economic analysis

It remains to be seen for which categories of chemicals biohydroxylation and epoxidation processes will prove to be economically viable. A detailed cost calculation for various potential processes for the hydroxylation of octane to octanol was prepared by Mathys [32*]. For a more general cost estimation one can make use of a rule of thumb that assumes the cost of running an (existing) large bioreactor to be around 50 \$ m⁻³ day⁻¹. Using this value, and the assumption that average costs for the generation of cell mass are 2.5 \$ (kg dry wt)⁻¹, we have calculated the minimum cost per kilogram product (molecular weight 150 kDa) for a number of hypothetical biocatalysts and process conditions (Table 3). This table shows that continuous processes in which cell

growth/induction are combined with biotransformation are preferable from the economic point of view, partially because the costs for the generation of cell mass decrease at relatively low dilution rates. From Table 3, we conclude that, for bulk processes, biotransformation costs well below 1 \$ kg⁻¹ product may become feasible in the future. However, much of the total process costs will often result from product recovery. For this cost-factor, the final product concentration is the most important determinant. Therefore, important future research challenges will be the extension of the half-life of the oxygenase involved and the decrease in its sensitivity (and that of the host strain) to product inhibition.

Conclusions

Presently, the integration of oxygenases in synthetic processes for fine-chemicals is hindered by the instability of these enzymes, the vulnerability of the microbial host cells, and the special skills required for handling these biocatalysts. Practical challenges include the development of robust host strains with low endogenous oxygen respiration rates, high NADH regeneration rates, a long oxygenase half-life (stable activity for a prolonged period of time), combined with a low sensitivity of the host and the enzyme to organic solvents and product inhibition. Large-scale biotransformation costs below 1 \$ per kg product will come within reach when these conditions have been met to a sufficient degree.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Muralidhar RV, Marchant R, Nigam P: **Lipases in racemic resolutions.** *J Chem Technol Biotechnol* 2001, **76**:3-8.
 2. Holland HL, Weber HK: **Enzymatic hydroxylation reactions.** *Curr Opin Biotechnol* 2000, **11**:547-553.
- Overview of mechanistic studies, substrate recognition, enzyme manipulation and novel enzymes in the field of hydroxylations.

3. Nesheim JC, Lipscomb JD: Large kinetic isotope effects in methane oxidation catalyzed by methane monooxygenase: evidence for C-H bond cleavage in a reaction cycle intermediate. *Biochemistry* 1996, **35**:10240-10247.
4. Staijen IE, van Beilen JB, Witholt B: Expression, stability and performance of the three-component alkane monooxygenase of *Pseudomonas oleovorans* in *Escherichia coli*. *Eur J Biochem* 2000, **267**:1957-1965.
One of a few studies exploring the potential reasons for the lower specific activities of a hydroxylase after expression in *E. coli*.
5. Panke S, De Lorenzo V, Kaiser A, Witholt B, Wubbolts MG: Engineering of a stable whole-cell biocatalyst capable of (S)-styrene oxide formation for continuous two-liquid phase applications. *Appl Environ Microbiol* 1999, **65**:5619-5623.
6. Duetz WA, Wind B, van Andel JG, Barnes MR, Williams PA, Rutgers M: Biodegradation kinetics of toluene, *m*-xylene, *p*-xylene and their intermediates through the upper TOL pathway in *Pseudomonas putida* (pWW0). *Microbiology* 1998, **144**:1669-1675.
7. Nölthe C, Hartmans S: Formation and degradation of styrene oxide stereoisomers by different microorganisms. *Biocatalysis* 1994, **10**:219-225.
8. Panke S, Witholt B, Schmid A, Wubbolts MG: Towards a biocatalyst for (S)-styrene oxide production: characterization of the styrene degradation pathway of *Pseudomonas* sp. strain VLB120. *Appl Environ Microbiol* 1998, **64**:2032-2043.
9. Murdock D, Ensley BD, Serdar C, Thalen M: Construction of metabolic operons catalyzing the *de novo* biosynthesis of indigo in *Escherichia coli*. *Biotechnology* 1993, **11**:381-386.
10. Hugo N, Armengaud J, Gaillard J, Timmis KN, Jouanneau Y: A novel [2Fe-2S] ferredoxin from *Pseudomonas putida* mt-2 promotes the reductive reactivation of catechol 2,3-dioxygenase. *J Biol Chem* 1998, **273**:9622-9629.
11. Staijen IE, Hatzimanikatis V, Witholt B: The AlkB monooxygenase of *Pseudomonas oleovorans* – synthesis, stability and level in recombinant *Escherichia coli* and the native host. *Eur J Biochem* 1997, **244**:462-470.
12. Shaler TA, Klecka GM: Effects of dissolved oxygen concentration on biodegradation of 2,4-dichlorophenoxyacetic acid. *Appl Environ Microbiol* 1986, **51**:950-955.
13. Longmuir IS: Respiration rate as a function of oxygen concentration. *Biochem J* 1954, **57**:81-87.
14. Trilli A: Scale-up of fermentations. In *Manual of Industrial Microbiology and Biotechnology*. Edited by Demain AL, Solomon NA. American Society for Microbiology, Washington, DC; 1986:277-307.
15. Rols JL, Condoret JS, Fonade C, Goma G: Mechanism of enhanced oxygen transfer in fermentation using emulsified oxygen vectors. *Biotechnol Bioeng* 1990, **35**:427-435.
16. De Smet MJ, Kingma J, Witholt B: The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim Biophys Acta* 1978, **506**:64-80.
17. Pedrotta V, Witholt B: Isolation and characterization of the *cis-trans*-unsaturated fatty acid isomerase of *Pseudomonas oleovorans* GPo12. *J Bacteriol* 1999, **181**:3256-3261.
18. Kieboom J, Dennis JJ, de Bont JAM, Zylstra GJ: Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. *J Biol Chem* 1998, **273**:85-91.
This paper shows the existence of efflux pumps that remove organic solvents from the cytoplasmic membrane. These efflux systems show a striking resemblance to proton-dependent multidrug efflux systems.
19. Ramos JL, Duque E, Godoy P, Segura A: Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J Bacteriol* 1998, **180**:3323-3329.
20. Schmid A, Kollmer A, Mathys RG, Witholt B: Development toward large-scale bacterial bioprocesses in the presence of bulk amounts of organic solvents. *Extremophiles* 1998, **2**:249-256.
21. Duetz WA, Ruedi L, Hermann R, O'Connor K, Buchs J, Witholt B:
 - Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Appl Environ Microbiol* 2000, **66**:2641-2646.
 Description of a system for the miniaturized growth of large culture collections. Because of the high oxygen-transfer rates the system is suitable for screening for new oxygenases.
22. Minas W, Bailey JE, Duetz W: Streptomycetes in micro-cultures: growth, production of secondary metabolites, and storage and retrieval in the 96-well format. *Antonie van Leeuwenhoek Int J* 2000, **78**:297-305.
23. Dalbøge H, Lange L: Using molecular techniques to identify new microbial catalysts. *Trends Biotechnol* 1998, **16**:265-272.
General approach to use phylogenetic data and DNA sequences to discover new members of particular enzyme families.
24. Smits THM, Röthlisberger M, Witholt B, van Beilen JB: Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ Microbiol* 1999, **1**:307-318.
25. Okuta A, Ohnishi K, Harayama S: PCR isolation of catechol 2,3-dioxygenase gene fragments from environmental samples and their assembly into functional genes. *Gene* 1998, **212**:221-228.
26. Wang RF, Wennerstrom D, Cao WW, Khan AA, Cerniglia CE: Cloning, expression, and characterization of the *katG* gene, encoding catalase-peroxidase, from the polycyclic aromatic hydrocarbon-degrading bacterium *Mycobacterium* sp strain PYR-1. *Appl Environ Microbiol* 2000, **66**:4300-4304.
27. Chang PK, Yu JJ, Ehrlich KC, Boue SM, Montalbano BG, Bhatnagar D, Cleveland TE: *adhA* in *Aspergillus parasiticus* is involved in conversion of 5-hydroxyaverantin to averufin. *Appl Environ Microbiol* 2000, **66**:4715-4719.
28. Snyder MJ, Scott JA, Anderson JF: Sampling P450 diversity by cloning polymerase chain reaction products obtained with degenerate primers. *Methods Enzymol* 1996, **272**:304-312.
29. Souter A, McLean KJ, Smith WE, Munro AW: The genome sequence of *Mycobacterium tuberculosis* reveals cytochromes P450 as novel anti-TB drug targets. *J Chem Technol Biotechnol* 2000, **75**:933-941.
30. Sakaki T, Inoue K: Practical application of mammalian cytochrome P450. *J Biosci Bioeng* 2000, **90**:583-590.
31. Miles CS, Ost TWB, Noble MA, Munro AW, Chapman SK: Protein engineering of cytochromes P-450. *Biochim Biophys Acta – Protein Struct Mol Enzym* 2000, **1543**:383-407.
32. Mathys RG, Schmid A, Witholt B: Integrated two-liquid phase bioconversion and product-recovery processes for the oxidation of alkanes: process design and economic evaluation. *Biotechnol Bioeng* 1999, **64**:459-477.
A detailed cost analysis of a potential large-scale process for the use of alkane hydroxylase for the industrial production of medium-priced chemicals.
33. Li Z, Feiten HJ, van Beilen JB, Duetz W, Witholt B: Preparation of optically active N-benzyl-3-hydroxypyrrolidine by enzymatic hydroxylation. *Tetrahedron Asymmetry* 1999, **10**:1323-1333.
34. Jiang HY, Parales RE, Lynch NA, Gibson DT: Site-directed mutagenesis of conserved amino acids in the α subunit of toluene dioxygenase: potential mononuclear non-heme iron coordination sites. *J Bacteriol* 1996, **178**:3133-3139.
35. Kiener A: Enzymatic oxidation of methyl groups on aromatic heterocycles: a versatile method for the preparation of heteroaromatic carboxylic acids. *Angew Chem Int Ed Engl* 1992, **31**:774-775.
36. Duetz WA, Fjallman AHM, Ren S, Jourdat C, Witholt B: Biotransformation of *D*-limonene to (+) *trans* carveol by toluene-grown *Rhodococcus opacus* PWD4 cells. *Appl Environ Microbiol* 2001, **67**: 2829-2832.
37. Fulco AJ, Kim BH, Matson RS, Narhi LO, Ruettinger RT: Nonsubstrate induction of soluble cytochrome P-450 monooxygenase by phenobarbital. *Mol Cell Biochem* 1983, **53-54**:155-162.
38. Capdevila JH, Wei SZ, Helvig C, Falck JR, Belosludtsev Y, Truan G, Graham Lorence SE, Peterson JA: The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3. *J Biol Chem* 1996, **271**:22663-22671.

39. Oliver CF, Modi S, Sutcliffe MJ, Primrose WU, Lian LY, Roberts GCK: A single mutation in cytochrome P450 BM3 changes substrate orientation in a catalytic intermediate and the regioselectivity of hydroxylation. *Biochemistry* 1997, **36**:1567-1572.
40. Harford-Cross CF, Carmichael AB, Allan FK, England PA, Rouch DA, Wong LL: Protein engineering of cytochrome P450cam (CYP101) for the oxidation of polycyclic aromatic hydrocarbons. *Protein Eng* 2000, **13**:121-128.
41. Jones JP, O'Hare EJ, Wong LL: Oxidation of polychlorinated benzenes by genetically engineered CYP101 (cytochrome P450_{cam}). *Eur J Biochem* 2001, **268**:1460-1467.
42. Betlach MC, Kealey JT, Ashley GW, McDaniel R: Characterization of the macrolide P-450 hydroxylase from *Streptomyces venezuelae* which converts narbomycin to picromycin. *Biochemistry* 1998, **37**:14937-14942.
43. Walczak RJ, Dickens ML, Priestley ND, Strohl WR: Purification, properties, and characterization of recombinant *Streptomyces* sp. strain C5 DoxA, a cytochrome P-450 catalyzing multiple steps in doxorubicin biosynthesis. *J Bacteriol* 1999, **181**:298-304.
44. Minoletti C, Dijols S, Dansette PM, Mansuy D: Comparison of the substrate specificities of human liver cytochrome p450s 2C9 and 2C18: application to the design of a specific substrate of CYP2C18. *Biochemistry* 1999, **38**:7828-7836.
45. Tsao CC, Wester MR, Ghanayem B, Coulter SJ, Chanas B, Johnson EF, Goldstein JA: Identification of human CYP2C19 residues that confer S-mephenytoin 4'-hydroxylation activity to CYP2C9. *Biochemistry* 2001, **40**:1937-1944.
46. Shultz MA, Morin D, Chang AM, Buckpitt A: Metabolic capabilities of CYP2F2 with various pulmonary toxicants and its relative abundance in mouse lung subcompartments. *J Pharmacol Exp Ther* 2001, **296**:510-519.
47. Mancy A, Antignac M, Minoletti C, Dijols S, Mouries V, Duong NTH, Battioni P, Dansette PM, Mansuy D: Diclofenac and its derivatives as tools for studying human cytochromes P450 active sites: particular efficiency and regioselectivity of P4502Cs. *Biochemistry* 1999, **38**:14264-14270.
48. Bell-Parikh LC, Guengerich FP: Kinetics of cytochrome P450 2E1-catalyzed oxidation of ethanol to acetic acid via acetaldehyde. *J Biol Chem* 1999, **274**:23833-23840.
49. Chen DW, Frey PA: Phenylalanine hydroxylase from *Chromobacterium violaceum*: tetrahydropterin oxidase activity and the role of iron in hydroxylation. *FASEB J* 1998, **12**:315.
50. Wackett LP: Toluene dioxygenase from *Pseudomonas putida* F1. *Methods Enzymol* 1990, **188**:39-45.
51. Fox BG, Froland WA, Dege JE, Lipscomb JD: Methane monooxygenase from *Methylosinus trichosporium* OB3b: purification and properties of a three-component system with high specific activity from a type II methanotroph. *J Biol Chem* 1989, **264**:10023-10033.
52. Imbeault NYR, Powlowski JB, Colbert CL, Bolin JT, Eltis LD: Steady-state kinetic characterization and crystallization of a polychlorinated biphenyl-transforming dioxygenase. *J Biol Chem* 2000, **275**:12430-12437.
53. Suske WA, Held M, Schmid A, Fleischmann T, Wubboldts MG, Kohler HPE: Purification and characterization of 2-hydroxybiphenyl 3- monooxygenase, a novel NADH-dependent, FAD-containing aromatic hydroxylase from *Pseudomonas azelaica* HBP1. *J Biol Chem* 1997, **272**:24257-24265.
54. Pikus JD, Studts JM, McClay K, Steffan RJ, Fox BG: Changes in the regioselectivity of aromatic hydroxylation produced by active site engineering in the diiron enzyme toluene 4-monooxygenase. *Biochemistry* 1997, **36**:9283-9289.
55. Ensley BD, Gibson DT: Naphthalene dioxygenase – purification and properties of a terminal oxygenase component. *J Bacteriol* 1983, **155**:505-511.
56. Newman LM, Wackett LP: Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. *Biochemistry* 1995, **34**:14066-14076.
57. Reddy CC, Vaidyanathan CS: Purification, properties and induction of a specific benzoate-4-hydroxylase from *Aspergillus niger* (UBC 814). *Biochim Biophys Acta* 1975, **384**:46-57.
58. Michalover JM, Ribbons DW: 3-Hydroxybenzoate 4-hydroxylase from *Pseudomonas testosteroni*. *Biophys Res Commun* 1973, **55**:888-896.
59. Eppink MHM, Boeren SA, Vervoort J, VanBerkel WJH: Purification and properties of 4-hydroxybenzoate 1-hydroxylase (decarboxylating), a novel flavin adenine dinucleotide-dependent monooxygenase from *Candida parapsilosis* CBS604. *J Bacteriol* 1997, **179**:6680-6687.
60. Donoghue NA, Norris DB, Trudgill PW: The purification and properties of cyclohexanone oxygenase from *Nocardia globerula* CL1 and *Acinetobacter* NCIB 9871. *Eur J Biochem* 1976, **63**:175-192.
61. Neujahr HY, Kjellen KG: Phenol hydroxylase from yeast: reaction with phenol derivatives. *J Biol Chem* 1978, **253**:8835.
62. Griffin M, Trudgill PW: Purification and properties of cyclopentanone oxygenase of *Pseudomonas* NCIB 9872. *Eur J Biochem* 1976, **63**:199-209.
63. Gorlatova N, Tchorzewski M, Kurihara T, Soda K, Esaki N: Purification, characterization, and mechanism of a flavin mononucleotide-dependent 2-nitropropane dioxygenase from *Neurospora crassa*. *Appl Environ Microbiol* 1998, **64**:1029-1033.
64. Kido T, Tanizawa K, Inagaki K, Yoshimura T, Ishida M, Hashizume K, Soda K: 2-Nitropropane dioxygenase from *Hansenula mrakii* characterization of the enzyme and oxidation of anionic nitroalkanes. *Agric Biol Chem* 1984, **48**:2549-2554.
65. Fujisawa H, Hayaishi O: Protocatechuate 3,4-dioxygenase: I. Crystallization and characterization. *J Biol Chem* 1968, **243**:2673-2681.
66. Harpel MR, Lipscomb JD: Gentsiate 1,2-dioxygenase from *Pseudomonas* – substrate coordination to active-site Fe²⁺ and mechanism of turnover. *J Biol Chem* 1990, **265**:22187-22196.
67. Nakai C, Hori K, Kagamiyama H, Nakazawa T, Nozaki M: Purification, subunit structure, and partial amino acid sequence of meta pyrocatechase. *J Biol Chem* 1983, **258**:2916-2922.
68. Iwabuchi T, Harayama S: Biochemical and molecular characterization of 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7. *J Biol Chem* 1998, **273**:8332-8336.
69. Sanders SA, Williams CH, Massey V: The roles of two amino acid residues in the active site of L-lactate monooxygenase – mutation of arginine 187 to methionine and histidine 240 to glutamine. *J Biol Chem* 1999, **274**:22289-22295.
70. Zaborina O, Latus M, Eberspacher J, Golovleva LA, Lingens F: Purification and characterization of 6-chlorohydroxyquinol 1,2-dioxygenase from *Streptomyces rochei* 303 – comparison with an analogous enzyme from *Azotobacter* sp. strain-Gp1. *J Bacteriol* 1995, **177**:229-234.