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Oxygenase-Based Whole-Cell Biocatalysis in Organic Synthesis

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Molecules that cannot be superimposed on their mirror image are called chiral. The two possible configurations of chiral molecules, called enantiomers, differ in the three-dimensional orientation of some of their atoms. Thus, the two enantiomers may have different interactions with the active site of enzymes, receptors, and other biomolecules. For instance, (4R,4aS,6R)-(+)-nootkatone has a strong grapefruit odor and bitter taste, but the other enantiomer (4S,4aR,6S)-(−)-nootkatone has a weak woody odor and virtually no taste [44] (Fig. 1). Consequently, the chirality is very important in fine chemicals including vitamins, amino acids, and flavors as well as intermediates and building blocks for the synthesis of pharmaceuticals and agrochemicals.

Enzymes are very interesting catalysts for the synthesis of chiral compounds. They generally accept a wide range of complex molecules as substrates, and their reactions are highly enantio- and regioselective. Thus, enzymes can be used in simple and complex transformations without the need of blocking and deblocking steps that are common in the synthesis of chiral compounds. In addition, enzyme processes are very often cost-effective, flexible, clean, and sustainable [6]. Specifically, investment and manufacturing costs are lower, production utilities can be used flexibly for an entire range of products, the substrate spectrum is broader, waste production and energy consumption are lower, renewable resources are more applicable, and raw material requirements are lower in bioprocesses than in their chemical counterparts. Moreover, biocatalysis products are sometimes distinctive, of high quality, and can be registered as natural products. For instance, the production of enantiopure (S)-chloropropionic acid was recently switched from a chemical to a biological process [41]. The bioconversion is performed under mild reaction conditions in the absence of toxic raw materials, resulting in ten-fold reduced waste. Along the same lines, the Lonza group demonstrated that a biological L-carnitine production process is more profitable than a chemical counterpart [11]. The bioprocess yielded L-carnitine with almost 100% enantiomeric excess (e.e.). Additionally, the process was ecologically superior. Many examples have been listed in review articles [14, 108, 121, 125].

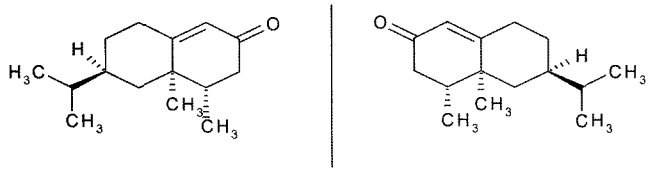

Fig. 1. Mirror images of enantiomers (4S,4aR,6S)-(−)-nootkatone (left) and (4R,4aS,6R)-(+)-nootkatone (right).

Biocatalytic oxidation of hydrocarbons such as readily available petrochemicals (*e.g.*, styrene, xylene, octane) and renewable plant oils (*e.g.*, limonene, valencene) is one of the most valuable transformations for synthetic applications [22]. Chemical counterparts are not competitive because of side reactions and thus have low product yield resulting in high costs for the product recovery. The importance of enantioselective oxidative catalysts has been recognized by the award of the Nobel Prize 2001 (Chemistry) to B. Sharpless for developing selective and industrially competitive oxidation technologies.

This review will focus on recent studies on the development of microbial processes for the selective oxidation of hydrocarbons; that is, from biocatalyst construction to bioprocess engineering.

Oxygenases as Catalysts

Asymmetric oxidation of organic compounds can be mediated by oxidoreductases such as haloperoxidases [1] and oxygenases [14]. Peroxidases oxidize a wide range of olefins with large

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e.e., are cofactor independent, and use H_2O_2 or organic peroxides as oxidants. However, the enzymes are subject to oxidant-mediated inactivation during biocatalysis, reducing their catalytic values for practical applications [1]. Here, we concentrate on the use of oxygenases as catalysts for asymmetric oxidation of hydrocarbons (*e.g.*, styrene epoxidation).

Since the early 1960s, oxygenase biocatalysis has been extensively investigated. A number of oxygenases were isolated from *Pseudomonas* sp. strains, and their catalytic mechanisms were characterized [126]. Most oxygenases are cofactor (NAD(P)H) dependent, and are multiple component enzymes with additional proteins for transport of electrons from NAD(P)H to the oxidation component. Thus, these enzymes have been classified in three groups according to the types of electron transfer systems [82]; heme oxygenases, non-heme iron oxygenases, and flavin oxygenases.

Heme Oxygenases

Heme oxygenases usually belong to the class of cytochrome P450 enzymes. These are cofactor (NAD(P)H) dependent, and multi-component enzymes. The electrons are transferred from NAD(P)H to the terminal cytochrome P450 component *via* typical electron transfer proteins (*i.e.*, a ferredoxin reductase and a ferredoxin). An exception is cytochrome P450 BM-3 of *Bacillus megaterium*, which consists of a single polypeptide with a P450 domain and an electron transport domain of the microsomal type [75].

The heme group of P450 oxygenases is directly involved in the oxidation process; the resting enzyme [low-spin, hexacoordinate, ferric form I (Fig. 2)] binds substrate (RH) reversibly resulting in a high-spin, pentacoordinate ferric complex II. An electron is transferred from NAD(P)H via the electron transfer chain to P450 to give an iron (II) species (the high-spin, pentacoordinate ferrous complex III). Reversible binding of oxygen then takes place to give the superoxide-iron complex (a semistable low-spin, hexacoordinate ferrous-O_2 adduct IV). Species I-IV of the P450 cycle and the low-spin, hexacoordinate ferrous-CO inhibitor complex V were isolated and characterized [3]. It is hypothesized that such species as a low-spin, ferric peroxycomplex VI, and oxyferryl (FeIV=O) intermediate VII, are further formed. Oxygen atom transfer from VII to the substrate yields an oxidized product (ROH) and regenerates state I. In the presence of external oxygenation agents, such as peracids (XOOH), the complex III may directly yield state VII via a shunt pathway (Fig. 2).

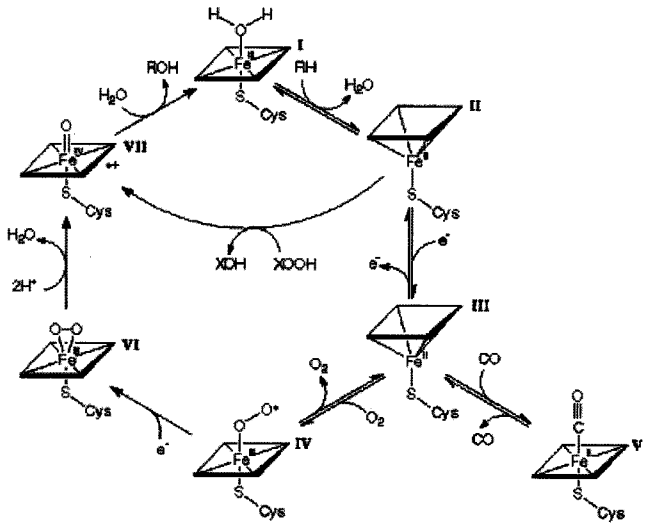

Fig. 2. Reaction cycle of cytochrome P450 oxygenases [3].
The picture was adopted from http://metallo.scripps.edu/promise/MAIN.html.

The cytochrome P450 oxygenases, which are originally involved in biosynthesis of steroids and polyketides [67], fatty acid metabolism, and detoxification of xenobiotics, catalyze a wide variety of reactions, including hydroxylation of carbon and heteroatoms and epoxidation of olefins as well as reductive dehalogenation and dealkylation of amines and ethers. For example, *Pseudomonas putida* cytochrome $P450_{cam}$ (camphor monooxygenase) catalyzes hydroxylation of the natural substrate camphor and several substrate analogs such as norcamphor and 1-methyl-norcamphor [8]. It also oxidizes styrene into (S)-styrene oxide with an e.e. of 66% [33].

Non-Heme Iron Oxygenases

Non-heme iron oxygenases, containing non-heme iron (*e.g.*, iron-sulfur complex) instead of heme as cofactor, catalyze hydroxylation, dioxygenation, and lipoxygenation. The alkane hydroxylase of *P. putida* GPo1 and xylene monooxygenase of *P. putida* mt-2 are the most intensively studied in the non-heme iron monooxygenase group. The alkane hydroxylase of *P. putida* GPo1 consists of a membrane-bound hydroxylase component (AlkB), and soluble electron transfer components; rubredoxin (AlkG) and rubredoxin reductase (AlkT) [129]. The AlkGT correspond to ferredoxin and ferredoxin reductase of heme oxygenases, respectively. The AlkB hydroxylase contains a diiron cluster that is oxo-bridged and might be coordinated by the nitrogen atoms of eight histidines [109]. An iron-oxo species was proposed to attack the substrate carbon bond and to form intermediates with a partial radical and cationic character [34, 35]. Two electrons required to activate molecular oxygen are transferred from NADH to the diiron cluster of AlkB via the electron transfer components AlkGT. The alkane hydroxylase shows a wide substrate spectrum, including hydroxylation of alkanes, epoxidation of terminal alkenes, sulfoxidation, and demethylation [34, 63, 128].

The xylene monooxygenase of *P. putida* mt-2 is composed of a membrane-bound oxygenase component (XylM) and a soluble NADH:acceptor reductase (XylA) [111, 112]. XylM shows 25% amino acid sequence identity with AlkB including the eight-histidine motif [110, 123]. The xylene monooxygenase oxidizes methyl- as well as vinyl-groups

of alkanes including styrene and substituted styrene derivatives [46, 138]. For instance, pseudocumene was efficiently transformed into 3,4-dimethylbenzaldehyde via 3,4-dimethylbenzalcohol [15], and styrene was epoxidized to (S)-styrene oxide with an e.e. of 92% [137].

The naphthalene dioxygenase, a representative of aromatic dioxygenases, also belong to the non-heme iron oxygenase group. It consists of an iron-sulfur flavoprotein reductase, an iron-sulfur ferredoxin, and a catalytic oxygenase component [122]. The oxidation component is composed of large and small subunits, α and β, respectively, that are in an $\alpha_3\beta_3$ configuration. Each alpha subunit contains a Rieske-type [^{2}Fe-^{2}S] center in which enzyme activity depends on the presence of mononuclear iron. The naphthalene dioxygenase usually introduces two oxygen atoms into unfunctionalized atoms with high enantioselectivity yielding cis-diols. The dioxygenase has activity for not only naphthalene and its derivatives, but also fluorene, dibenzofuran, dibenzothiophene, 9,10-dihydroanthracene, and 9,10-dihydrophenanthrene [98, 99]. The enzyme is also able to catalyze monohydroxylation of aromatic compounds (*e.g.*, 1-indanone, 2-indanone) and N-heterocycles (*e.g.*, carbazole).

Flavin Oxygenases

Flavin oxygenases, which use FAD instead of iron complexes as cofactors [47, 85], are known to catalyze epoxidation, aromatic oxygenation, heteroatom oxygenation, and Bayer-Villiger oxidation (oxygen insertion reaction [21, 72]). Styrene monooxygenases to catalyze styrene epoxidation are the most intensively investigated in the flavin oxygenase group; the styrene monooxygenases, isolated from *Pseudomonas* sp., are composed of a soluble epoxidizing component (StyA) and a soluble electron transfer protein (StyB). A catalytic mechanism has been proposed: StyB transfers electrons from NADH to FAD and makes them available for oxygen activation by StyA. Steady state kinetic data indicate a sequential binding mechanism of NADH and FAD for StyB [80]. Neither purified StyA nor StyB contains a tightly bound flavin [79]. The styrene monooxygenases of *Pseudomonas* sp. VLB120 and *P. fluorescens* ST epoxidize styrene to (S)-styrene oxide with an e.e. of over 99% and show a broad substrate spectrum [9, 107]. In addition, the styrene monooxygenases of *Pseudomonas* sp. VLB120 have high sequence identities (in the range of 95 and 98%) with the StyA of *P. putida* S12, *P. putida* CA-3, *Pseudomonas* sp. strain Y2, *P. fluorescens* ST [79], and *P. putida* SN1 [90].

BIOCATALYST CONSTRUCTION

A cell-free application of oxygenases would significantly increase the synthetic value of oxygenases since the setup is simpler as compared with a whole-cell system. However, biocatalytic oxidations are usually cofactor dependent and catalyzed by multi-component, and/or membrane-bound enzymes. Thus, living whole-cell cultures are favored in practical applications over the use of isolated enzymes.

The catalytic efficiency of a bacterial cell as a biocatalyst is determined by the maximal enzyme activity, mass transfer efficiency, and cofactor regeneration capacity. Besides this, the catalytic efficiency may be influenced by the regulatory environment in the cells (*e.g.*, catabolite repression), byproduct formation, and product degradation. Here, we will describe properties of wild-type and recombinant strains as biocatalysts for asymmetric oxidation of hydrocarbons.

Wild-Type Strain Based Biocatalysts

Wild-type strains, which are able to assimilate organic chemicals as carbon source, often have oxidation activities over 100 U/g CDW [29], and are rather stable in the presence of those compounds [95]. As a result, wild-type strains are often used as biocatalysts for the production of metabolic intermediates or metabolite mimics, when these are sought as fine chemicals. For instance, *P. putida* is used for the production of 5-methyl-2-pyrazinic acid from 2,5-dimethylpyrazine [11]. 5-Methyl-2-pyrazinic acid, a mimic of 5-methylbenzoic acid, is produced by enzymes involved in p-xylene degradation (Fig. 3A).

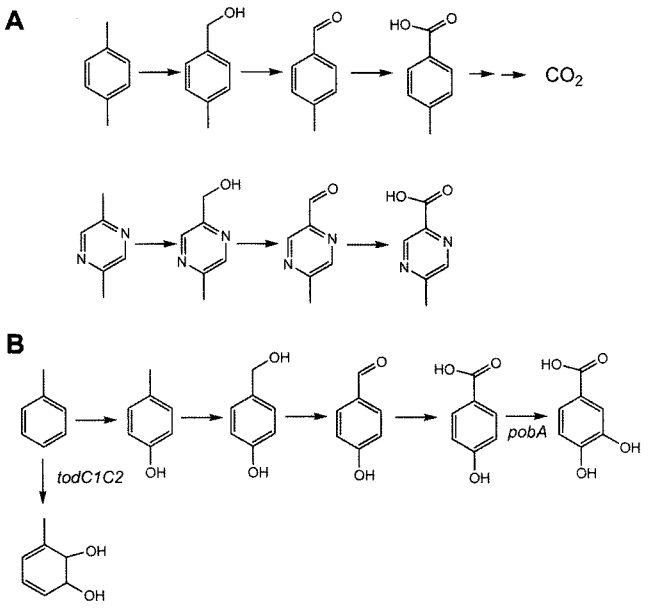

Fig. 3. A. Biotransformation of 2,5-dimethylpyrazin to 5-methyl-2-pyrazinic acid by *P. putida*. 5-Methyl-2-pyrazinic acid, a mimic of 5-methylbenzoic acid, is produced as the end product by the enzymes involved in p-xylene degradation; **B.** Biotransformation of toluene to p-hydroxybenzoate by *P. putida* DOT-T1E. The *todC1C2* genes encoding toluene dioxygenase and the *pobA* gene were knocked out to prevent side reactions.

Wild-type strains however have some drawbacks as catalysts. They have versatile enzymes and regulatory systems involved in the assimilation of hydrocarbons. This may cause product degradation, byproduct formation, and catabolite repression [104, 117]. When the product is degraded or byproducts are produced by host enzymes, it is necessary to knock out genes for the enzymes, for example by mutagenesis. This was illustrated in the engineering of *P. putdia* DOT-T1E to accumulate p-hydroxybenzoate from toluene [94]. The *todC1C2* genes encoding the α and β subunits of toluene dioxygenase, which catalyzes the first step in the toluene degradation pathway, was deleted by homologous recombination (Fig. 3B). The *pobA* gene, whose product is involved in degradation of the product, was also knocked out. In some cases, blocking of specific pathways may be difficult owing to the presence of multiple enzyme activities.

Besides, this catabolite repression may restrict the use of wild-type strains as catalysts. A number of non-aromatic compounds such as organic acids and carbohydrates have been shown to repress the degradation of aromatic compounds. Specifically, induction of the *styA* promoter in *P. fluorescens* ST was repressed in the presence of glucose [9].

Other issues in the use of wild-type strains as biocatalysts may include demanding cultivation, poor growth on minimal media, and suspected pathogenicity of the strain of interest.

Recombinant Biocatalysts

E. coli or *P. putida* based recombinant strains can be used as alternatives to wild-type strains. *P. putida* recombinants could be useful for biotransformations in the presence of toxic chemicals. A number of solvent tolerant *P. putida* strains such as S12 [132], DOT-T1E [96], and IH-2000 [55] have been isolated and used as hosts for the expression of oxygenase genes [83, 134]. However, the long-term biotransformation rates achieved with these strains are lower than 20 U/l despite high intracellular enzyme activities [52, 83, 134] (Table 1). One example is the

Table 1. Microbial hydroxylation and epoxidation processes[a].

Substrate[b]	Product[c]	Microorganism[d]	Scale[e]	2nd liquid phase[b,e]	Max sp. activity[e] [U/g CDW]	Cell dry weight[e] [g/l_{aq}]	Avg (max) activity[e] [U/l_{tot}]	Product concn[e] [mM]	Process type[f]	Year (reference)
Hydroxylations										
PGT	PGTox	*A. ochraceus*	0.25 l	OA	2.1	3	2.8 (6.3)	7	H	1988 [18]
Benzene	BCG	*Pseudomonas*	0.9 l	n.a.	45	0.3	14	9.6	G	1988 [130]
C8	C8OH	*P. putida*[g]	0.6 l	C8	60	1	20	7.7	H	1992 [10]
NAP	NCG	*P. putida*	75 ml	C12	104	0.4	25	7.5	H	1992 [45]
C8	C7COOH	*E. coli*[g]	0.65 l	C8	25	2.3	58	11	G	1993 [31]
2,5-DMP	MPCOOH	*P. putida*	20 l	n.a.	n.m.	n.m.	45	150	C	1995 [61]
C8	C7COOH	*E. coli*[g]	1.5 l	C8	4	42	130 (170)	46	C	1996 [136]
Toluene	**TCG**	***P. putida***	**1.1**	**C14**	**n.m.**	**>10**	**880**	**450**	**C**	**1996 [70]**
F-benzene	FC	*P. putida*	2.5 l	n.a.	23	6	140	120	I	1996 [70]
C8	C8OH	*E. coli*[g]	3 l	C8	13	5.2	66	13	G	1998 [102]
Toluene	TCG	*E. coli*[g]	1.25 l	n.a.	n.m.	12	29 (44)	21	C	1999 [91]
Indene	Indandiol	*E. coli*[g]	12 l	SBO	n.m.	n.m.	13	6.4	C	1999 [97]
2-PP	2-PPOH	*E. coli*[g]	2 l	n.a.	5.4	6.5	35	31	I	1999 [48]
Toluene	3-MC	*P. putida*[g]	0.8 l	Octanol	n.m.	n.m.	7.5	48	C	2001 [52]
Limonene	(+) Carveol	*R. opacus*	5 ml	n.a.	15	1.1	9 (16)	1.2	D	2001 [28]
BH	OBO	*E. coli*[g]	0.1 l	n.a.	n.m.	17	56	151	I	2001 [116]
Toluene	3-MC	*P. putida*[g]	1.8 l	Octanol	n.m.	2.1	11	17	F	2002 [54]
Indene	Indandiol	*Rhodococcus*	4.5 l	n.a.	n.m.	30	24	8.4	C	2002 [2]
Eugenol	FA	*R. eutropha*	50 ml	n.a.	n.m.	n.m.	15 (48)	18	D	2002 [81]
BH	**OBO**	***E. coli***[g]	**1.5 l**	**n.a.**	**55**	**5**	**270**	**32**	**C**	**2002 [26]**
2-MQ	2-QCA	*P. putida*	14 l	n.a.	n.m.	n.m.	0.6	55	C	2002 [135]
Pseudocumene	**3,4-DMBA**	***E. coli***[g]	**2 l**	**BEHP**	**43**	**18**	**210 (360)**	**330**	**C**	**2003 [13]**
Pseudocumene	**3,4-DMBA**	***E. coli***[g]	**30 l**	**BEHP**	**30**	**20**	**160 (250)**	**270**	**J**	**2003 [13]**
Cinnamonitrile	DCAN	*E. coli*[g]	2 l	n.a.	1.0	8.2	4.3 (7.7)	2.9	C	2004 [139]
1,3-Dithiane	1,3-DTO	*E. coli*[g]	1 l	n.a.	n.m.	12	71	2.9	C	2004 [140]
m-Xylene	3-MC	*P. putida*	1 l	Decanol	n.m.	n.m.	18	70	C	2004 [100]
Cyclohexanone	ε-CL	*E. coli*[g]	1 l	n.a.	n.m.	46.6	50	10.2	C	2005 [68]
Benzonitrile	DCD	*E. coli*[g]	2 l	n.a.	2.9	16	14.3 (43)	10.7	C	2005 [139]
Limonene	PA	*P. putida*[g]	1.5 l	BEHP	n.m.	15	3.4	45	C	2005 [127]
Cyclohexanone	**ε-CL**	***E. coli***[j]	**1 l**	**n.a.**	**n.m.**	**42**	**140**	**134**	**C**	**2007 [69]**

Table 1. Continued.

Substrate[b]	Product[c]	Microorganism[d]	Scale[e]	2nd liquid phase[b,e]	Max sp. activity[e] [U/g CDW]	Cell dry weight[e] [g/l_{aq}]	Avg (max) activity[e] [U/l_{tot}]	Product concn[e] [mM]	Process type[f]	Year (reference)
Epoxidations										
C8ene	(R)-C8ox	*P. oleovorans*	1 l	C8ene	n.m.	n.m.	11	40	D	1981 [25]
C2ene	C2ox	*Mycob.* Py1	1.5 l	n.a.	25	n.m.	13	n.m.	A	1983 [23]
C14ene	C14ox	*N. corallina*	3 l	C14ene	3	15	44	400	C	1984 [37]
C3ene	C3ox	*Methylos.* sp.	8.5 ml	n.a.	15	2.4	35	n.m.	A	1984 [50]
C3ene	(R)-C3ox	*N. corallina*	6 ml	Paraffin	7.2	3	8.6 (22)	n.m.	B	1986 [73]
C3ene	**(R)-C3ox**	***N. corallina***	**2.6 l**	**n.a.**	**20**	**15**	**150 (280)**	**n.m.**	**C**	**1986 [36]**
C8ene	(R)-C8ox	*P. oleovorans*	50 ml	C8ene	n.m.	n.m.	33	n.m.	B	1986 [131]
C3ene	C3ox	*Mycob.* E3	1.7 l	C16	0.2	29	2.7 (5.8)	16	B	1987 [12]
2-MH	2-Mhox	*N. corallina*	20 l	C14	1.5	15	12	50	C	1989 [124]
C8ene	(R)-C8ox	*N. corallina*	85 ml	C16	3.4	7.5	12	100	B	1990 [59]
Styrene	(S)-Stox	*E. coli*[g]	1.5 l	C12	5	30	22 (37)	91	C	1996 [136]
APE	(S)-PGE	*Mycob.* M156	5 ml	C16	6	2.5	2.5 (7.5)	0.6	D	1998 [93]
1,7-C8ene	(R)-1-C8ox	*P. oleovorans*	50 ml	C7	11	2.5	30	240	F	1999 [27]
1,7-C8ene	(R)-1-C8ox	*P. oleovorans*	5 l	n.a.	34	0.4	15	n.m.	G	2000 [120]
Styrene	(S)-Stox	*E. coli*[g]	2 l	BEHP	50	15	150 (300)	180	C	2000 [86]
Styrene	(S)-Stox	*E. coli*[g]	30 l	BEHP	35	12	150	270	J	2002 [84]
Styrene	**(S)-Stox**	***E. coli*[g]**	**2 l**	**BEHP**	**60**	**32**	**630 (900)**	**310**	**C**	**2006 [88]**
Styrene	(S)-Stox	*P. putida*	3 l	BEHP	40	8.4	70 (160)	150	C	2006 [43]
Styrene	**(S)-Stox**	***Pseud.* sp.**	**2 l**	**BEHP**	**37**	**24**	**170 (320)**	**290**	**C**	**2007 [89]**

[a]Modified from [87].

[b]PGT, progesterone; C8, *n*-octane; NAP, naphthalene; 2,5-DMP, 2,5-dimethylpyrazine; F-benzene, fluorobenzene; 2-PP, 2-hydroxybiphenyl; BH, bicyclo[3.2.0]hept-2-en-6-one; 2-MQ, 2-methylquinoline; C8ene, 1-octene; C2ene, ethylene; C14ene, 1-tetradecene; C3ene, propylene; 2-MH, 2-methylhept-1-ene; APE, allylphenylether; 1,7-C8ene, 1,7-octadiene; OA, oleic acid; C12, *n*-dodecane; C14, tetradecene; SBO, soybean oil; BEHP, bis(2-ethyl hexyl)phthalate; C16, *n*-hexadecane; C7, *n*-heptane.

[c]PGTox, 11-hxdroxy progesterone; BCG, benzene-*cis*-glycol; C8OH, 1-octanol; NCG, naphthalene-*cis*-glycol; C7COOH, octanoic acid; MPCOOH, 5-methylpyrazinecarboxylic acid; TCG, toluene-*cis*-glycol; FC, fluorocatechol; 2-PPOH, 2,3-dihydroxybiphenyl; 3-MC, 3-methyl catechol; FA, ferulic acid; OBO, mixture of 2-oxabicyclo[3.3.0]oct-6-en-3-one and 3-oxabicyclo[3.3.0]oct-6-en-3-one; 2-QCA, 2-quinoxalinecarboxylic acid; 3,4-DMBA, 3,4-dimethylbenzaldehyde; DCAN, trans-3-[(5S,6R)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile; 1,3-DTO, 1,3-dithiane-1-oxide; ε-CL, ε-caprolactone; DCD, cis-1,2-dihydroxy-3-cyanocyclohexa-3,5-dine; PA, perillyl alcohol; C8ox, 1-epoxyoctane; C2ox, ethylene oxide; C14ox, 1-epoxytetradecane; C3ox, propylene oxide; 2-MHox, 1-epoxy-2-methylheptane; (S)-Stox, styrene oxide; PGE, phenylglycidylether; 1-C8ox, 7-epoxyoct-1-ene.

[d]*A. ochraceus*, *Aspergillus ochraceus*; *R. opacus*, *Rhodococcus opacus*; *R. eutropha*, *Ralstonia eutropha*; *Mycob.*, *Mycobacterium*; *N. corallina*, *Nocardia corallina*; *Methylos.*, *Methylosinus*.

[e]Concentration in organic phase in two-liquid phase and in aqueous phase in single phase; n.m., not mentioned and not accessible; n.a., not applicable.

[f]A, immobilized cells in gas-solid reactor; B, immobilized cells in two-liquid phase culture; C, fed-batch process in continuously stirred tank reactor (CSTR) with added carbon source but no other medium components; D, shaking flasks or tubes; E, resting cells (no carbon source added) in CSTR; F, continuous process in membrane reactor; G, continuous culture in CSTR; H, batch mode in CSTR; I, fed-batch mode in CSTR, *in situ* product removal by a solid phase, either in the reactor or in a separate compartment; J, fed-batch mode in pilot-scale.

[g]Recombinant system.

toluene oxidation to 3-methylcatechol by *P. putida* MC2, which overproduces toluene dioxygenase and toluene *cis*-dihydrodiol dehydrogenase, the first two enzymes in the toluene degradation pathway of *P. putida* F1. The bioconversion activity increased up to 240 U/g CDW in a glucose-mineral medium containing 2 mM toluene at a cell density of 0.04 g CDW/l [53]. However, the long-term bioconversion rates in a bioreactor remained below 20 U/g CDW [52, 54]. In addition, *P. putida* KT2440 carrying the xylene monooxygenase genes of *P. putida* mt-2 on the chromosome reached over 40 U/g CDW epoxidation activity in a whole-cell assay measured for 5 min. Yet, the biotransformation rates during continuous cultivation in a two-liquid phase system remained below 5 U/g CDW [83].

On the other hand, *E. coli* based biocatalysts often show volumetric productivities in excess of 150U/l (Table 1). An example is the production of (S)-styrene oxide from styrene. The use of the styrene monooxygenases of *Pseudomonas* sp. VLB120, an optimized expression system based on the *alk* regulatory system, glucose-based fed-batch cultivation, and the choice of bis(2-ethylhexyl)phthalate as organic carrier solvent present at a volume fraction of 50% (v/v) enabled maximal oxidation rates of over 60 U/g CDW and volumetric productivities of 900 U/l [88]. However, *E. coli* based biocatalysts tend to produce acetic acid during oxidative biotransformations [13, 88, 102], which reduces the extent of cofactor regeneration per g carbon source, and decreases the proton gradient over the cytoplasmic

membranes [5]. This may result in a decrease of the NADH regeneration capacity of cells under carbon limited conditions. In addition, the stable and functional expression of heterologous oxygenase genes in *E. coli* cells can be difficult because of the changed molecular environment of these enzymes in *E. coli*, as compared with the molecular environment in wild-type strains [29, 118]. This may influence gene expression, protein stability, the ratio of multiple components, and the formation of reactive oxygen species. For instance, the specific activity of alkane hydroxylase produced in recombinant *E. coli* W3110 was *ca.* 6-fold lower than that of wild-type strain *P. putida* GPo1 [118].

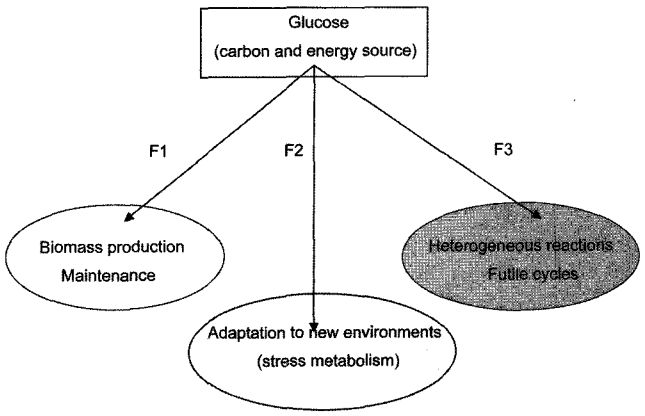

Fig. 4. Carbon balance during cofactor-dependent whole-cell oxidations.
The presence of organic substances (solvents, substrates, products) may place a metabolic burden on cells [32, 56]. Thus, a fraction of the carbon source (F2) is assumed to be used for host adaptation to and for maintenance of cell integrity in new environments.

BIOPROCESS ENGINEERING

The reported volumetric productivities of oxidative biotransformations are continuously increasing (Table 1). They did not depend on the chemical structure of products (aliphatic, aromatic, cyclic or saturated, unsaturated), the complexity of enzyme systems (electron transfer systems, number of components, interaction with membranes), or the bacterial species. However, in most cases, volumetric productivities remained below 200 U/l_{tot} despite enzyme activities that often exceed 100 U/g CDW. Furthermore, bioconversion rates are generally not stable over the entire reaction time during biotransformations [13, 88]. The relatively low long-term rates under process conditions and the decrease of bioconversion rates with time may be due to various factors including the toxic effects of organic substrates and products on host cells [49, 88, 137], transport limitations of apolar substrates and oxygen into the cells, the uncoupling of cofactor oxidation from substrate oxidation of oxygenases [66], or a low overall stability of recombinant biocatalysts [83, 118].

In this section, we describe the impact of cofactor regeneration, product toxicity, and mass transport on the biotransformation activity of oxygenase-based whole-cell biocatalysts.

Cofactor Regeneration

During oxidative biotransformations by growing cells, reduction equivalents (NAD(P)H) are used not only for biotransformation but also for cell growth (biomass production and maintenance such as protein turnover and transport of ions and nutrients) and cell adaptation to new environments containing toxic organic compounds and recombinant oxygenases (Fig. 4). Consequently, the availability of cofactors required for the oxidations may be influenced by various factors. Here, we describe cofactor regeneration and distribution in bacterial cells.

Balance between Anabolic and Catabolic Reactions. The carbon catabolism, which is involved in regeneration of cofactors (particularly NADH, ATP), is generally balanced with anabolism in bacterial cells [76]. Stimulation and repression of anabolic reactions are detected by two-component regulatory systems. The signals are transferred to transcription and/or protein activity regulators, resulting in a balance between catabolism and anabolism. There are a number of examples to support such a relationship. Depletion of essential nutrients or the presence of growth inhibitors triggered a decrease of the expression level of genes coding for proteins involved in catabolic reactions [19]. The specific oxygen uptake rate, which is a measure for the cofactor regeneration rate, is linear with respect to the specific growth rate of *E. coli* [17]. In addition, the flux ratio between glycolysis and the TCA cycle is not significantly influenced either by the overexpression of phosphofructokinase, pyruvate decarboxylase, pyruvate kinase, or by disruption of pyruvate kinase [30, 105].

Uncoupling of Anabolic and Catabolic Reactions. Catabolism can be uncoupled from anabolism. When microorganisms grow in excess of carbon sources, the energetic growth efficiency tends to be lower, which results from metabolic shifts of carbon or electron flow to less efficient pathways or energy-spilling reactions [103]. Poole and Haddock [92] noted that not all membrane-bound redox enzymes synthesized in *E. coli* are necessarily involved in energy conservation. When *E. coli* was grown under sulfate limitation, the NADH dehydrogenase became unable to transport protons. Besides this, an *E. coli* mutant defective in NADH dehydrogenase II (NDH-2), which results in $0H^+/e^-$, grew with greater efficiency as compared with the wild-type [17].

Futile Cycles and Cofactor-dependent Hydroxylations. There are various energy-spilling reactions referred to as futile cycles, which are not well understood. Futile cycles may result from leakage of ions (potassium and ammonium ions), weak organic acids or protons across the cell

membranes into the cytoplasm, thereby leading to a reduction of energy generation efficiency [103]. The cellular function of the futile cycles was supposed to allow cells to quickly adapt to changes in an environment [77]. Since heterologous oxidations are not involved in growth processes, these reactions can be considered as a kind of futile cycles. However, the difference is that the futile cycles do not repress significantly cell growth; in other words, their activities are more or less regulated to not exceed a certain extent. In contrast, heterologous oxidations are generally not under host control, strongly affecting the growth of host cells, as seen in the Case study below:

Case study: Oxygenation of pseudocumene by recombinant *E. coli* JM101 (pSPZ3)

Pseudocumene is oxidized to 3,4-dimethylbenzaldehyde via 3,4-dimethylbenzalcohol by the xylene monooxygenase, XylMA, of *P. putida* mt-2 [15]. One equivalent each of NADH and oxygen are used as reduction equivalent and oxidant respectively in each step. 3,4-Dimethylbenzaldehyde was produced by *E. coli* JM101 (pSPZ3) expressing *xylMA* in a fed-batch cultivation-based two-liquid phase system, consisting of bis(2-ethylhexyl)phthalate (BEHP) as an organic carrier solvent and an aqueous mineral medium [13]. During the bioconversion, the oxidation rate increased up to 90 U/g CDW, while the cell growth rate decreased gradually. After cell growth ceased, the oxidation rate decreased below 30 U/g CDW. Since the substrate and product concentrations remained in subtoxic ranges [16], the decrease of the growth rate might be due to the metabolic burden imposed by the cofactor consumption of XylMA (F3 in Fig. 4), and by overexpression of *xylMA*. In particular, the oxidation rate of 90 U/g CDW, which results in a NADH consumption rate of at least 90 μmol/g CDW/min, causes a significant metabolic burden. This burden can be estimated to be 55% of the cofactor regeneration rate required for cell growth at 0.09 h^{-1}, assuming a CO_2 evolution rate of 4.9 mmol/g CDW/h during cell growth at 0.09 h^{-1} [30], and the regeneration of 2 reduction equivalents for the formation of 1 CO_2 equivalent [58].

Overall, it is assumed that cofactor-dependent heterologous reactions may cause stress and hence repress the growth of host cells. The repression of cell growth may further decrease cofactor availability in cells, resulting in a reduction of cofactor-dependent biotransformation rates. Therefore, stable biocatalytic activity may be achieved via controlling bioconversion rates not to significantly inhibit cell growth, impairing cofactor regeneration with cell growth, and/or introducing an additional cofactor regeneration system that is not subject to the regulatory systems of host cells.

Increase of *In Vivo* Cofactor Regeneration Activity. One of the most successful approaches to increase the cofactor regeneration capacity of microbial cells was to introduce a foreign dehydrogenase into the microbes. For instance, the formate dehydrogenase gene was coexpressed with the gene of alanine dehydrogenase [38], leucine dehydrogenase [38, 42], or mannitol dehydrogenase [57] to provide NADH to the target reactions in recombinant *E. coli*. This enabled biotransformation rates of pyruvate into L-alanine and fructose into D-mannitol to reach over 70 and 200 U/g CDW, respectively. However, the use of formate dehydrogenase has been restricted for non-growing cells, probably because of transport limitation and/or toxicity of formate toward growing cells. There was no report, to our knowledge, to describe a remarkable improvement in cofactor (NADH) regeneration activity of growing cells.

Attenuation of Toxic Effects of Substrate and Products Toxicity

The majority of interesting substrates and products in oxidative biotransformations are poorly water-soluble (Table 1). These compounds accumulate in the cellular membranes, resulting in a swelling of the membrane bilayer and an increase of membrane fluidity. Finally, the membranes are permeabilized, impairing the function of the membranes as a matrix for embedded proteins and as a selective barrier [114]. For instance, a passive flux of protons and other ions across the cell membranes into the cytoplasm was detected in the presence of organic chemicals [74, 113, 115]. In addition, organic chemicals, particularly epoxides, were reported to react with biomolecules such as nicotinamide [78], 7-guanine, 3-adenine in double-stranded DNA [64], and cysteines, *e.g.*, in the active site of yeast alcohol dehydrogenases [62]. Overall, organic compounds may decrease the structural and functional integrity of cells, which is critical for cofactor regeneration and oxygenase synthesis.

There are many examples to show toxic effects of organic chemicals on the catalytic activities of whole-cell biocatalysts. Oxidation of 2-phenylphenol to 3-phenylcatechols [48], styrene epoxidation [88, 137], and indene oxidation to *cis*-indandiol [2] by whole-cell biocatalysts in fed-batch culture processes were limited by substrate and product toxicity. Thus, attenuation of substrate and product toxicity is critical to reach high biocatalytic activity.

One approach to overcome substrate and product toxicity is the use of two-liquid phase systems, which consist of an aqueous medium and an organic, water-immiscible solvent [119] (Fig. 5). The partitioning behavior of apolar substrates and products allows *in situ* extraction of the products, and regulation of the aqueous concentrations of the substrates. For instance, the use of a bis(2-ethylhexyl)phthalate-based two-phase system efficiently attenuated the toxicity of styrene and (S)-styrene oxide. The volumetric productivity and final product concentration increased up to 900 U/l_{tot} and 310 mM in the reaction medium, respectively [88].

A potential drawback of a two-liquid-phase system is the formation of stable emulsions, which may complicate

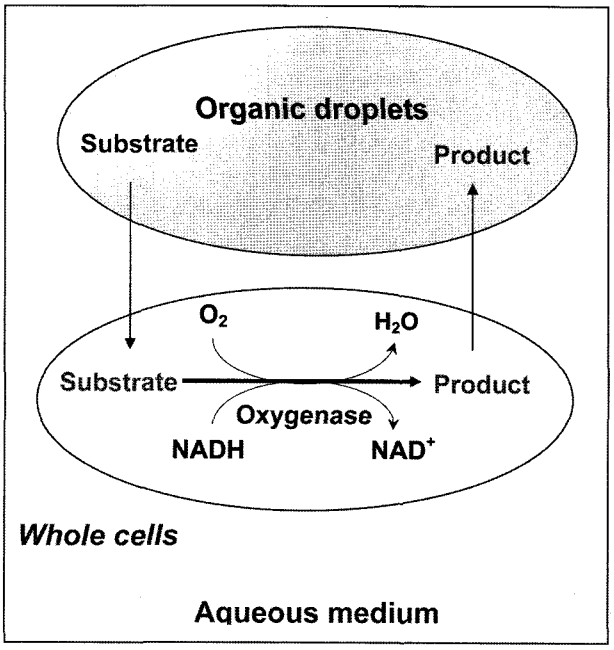

Fig. 5. Schematic diagram of a two-liquid-phase whole-cell oxidation system.
Reaction substrate is transferred to the cells, converted into product by intracellular oxygenases, and partitions to the organic phase. Oxygen in air serves as an oxidant. NAD(P)H is regenerated during catabolism of the carbon source. Aqueous concentrations of organic substrate and product are negligible because of high partition coefficients to the organic phase.

phase separation after biotransformations. The emulsion stability may be influenced by various factors including the presence of lipopolysaccharides produced by microorganisms and intracellular materials released from lysed cells, which act as surfactants. A possibility to avoid emulsification is to separate the two phases by membranes [27, 54]. The membranes may allow product removal via cross-flow filtration, which enables biocatalyst retention during continuous operation. However, mass transfer limitations and biofilm formation may impair the efficiency and scale-up of the processes based on membrane setup [24].

Another approach is to selectively separate the toxic products from the reaction medium via adsorbent resins. This concept is often coupled with controlled substrate feed rates, which are maintained at a level slightly below the maximal biooxidation rate. The adsorbent resins, which are based on polymeric matrices such as ion-exchange or hydrophobic resins, were used in separate columns [48, 70] or by direct mixing in the reaction medium [116]. The products are adsorbed on the resins thereby enabling *in situ* recovery. This system was applied for the production of 3-phenylcatechol [48], 3-tert-butylcatechol [71], and fluorobenzene-*cis*-glycol [70]. A limitation might be that this system needs a high circulation rate of the reaction medium for the efficient extraction of products (*e.g.*, 8.4 l reaction medium/l working volume/h for the extraction of 3-phenylcatechol). Thus, scale-up of the process based on separate columns may be difficult, and reactor operation costs could be high.

A different approach is to use solvent tolerant cells as biocatalysts or to increase the solvent tolerance of commonly used host strains. Solvent tolerant *Pseudomonas* sp. strains [51, 54, 83] (see section Recombinant biocatalysts for details) and *Ralstonia eutropha* [81] have been exploited as host cells for the production of toxic chemicals. Genome shuffling [39] or overexpression of genes involved in solvent tolerance [60, 133] have been investigated to increase the solvent tolerance of host strains.

Oxygen Transport

Oxygen transport is one of the key issues in oxidative biotransformation processes based on growing cells as biocatalysts. Oxygen limitation often takes place in high cell density cultures. The K_M values of many oxygenases for oxygen are in the range of 10 to 60 μM [106], which are one or two orders of magnitude higher than those of the electron transfer chain (below 1 μM [4]). Thus, the oxidation activities of whole-cell biocatalysts may decrease drastically under oxygen limitation.

Oxygen availability for the oxidations can be evaluated based on a linear relationship between the specific oxygen uptake rate for endogenous respiration and the specific growth rate of *E. coli* [17]. For example, assuming a cell concentration of 10 g CDW/l, and a growth rate of 0.07 h^{-1}, the cells will consume oxygen for endogenous respiration at a rate of 48 mmol/l/h. This means that a reactor able to provide an oxygen transfer rate of 90 mmol/l/h, which is usually available in large-scale reactors [29], may offer oxygen to the target reactions at 42 mmol/l/h, limiting maximal volumetric productivity to 700 U/l. Therefore, oxygen transfer and control of cell growth are critical for high volumetric productivity in whole-cell biocatalysis.

The oxygen transfer rate can be increased *via* various methods including application of a two-liquid-phase system coupled with hydrophobic organic solvents [101]. Rols *et al.* [101] showed that the use of *n*-dodecane as the organic solvent source resulted in an increase of the volumetric oxygen transfer coefficient (k_La) by 3.5-fold in a culture of *Aerobacter aerogenes*. As oxygen is 7.9 times more soluble in *n*-dodecane than in water, *n*-dodecane was suggested to act as an oxygen vector in the fermentation broth. Another example is the pseudocumene oxidation process in a two-phase system based on bis(2-ethylhexylphthalate) as the organic carrier solvent, which was described in the Case study. During biotransformation, the oxidation rate increased up to 1,500 U/l_{aq} in a 2-l scale reactor, indicating that oxygen was transferred to the oxygenases at a rate of 1,500 $\mu mol/l_{aq}/min$ (90 $mmol/l_{aq}/h$). During that time period, the cells grew to 16 g CDW/l at a rate of 0.14 h^{-1}, thereby consuming oxygen at a rate of

98 mmol/l_{aq}/h for endogeneous respiration. This indicates that the oxygen transfer rate from air to the cells may increase up to 188 mmol/l_{aq}/h in the two-liquid-phase system.

In addition to the application of a two-liquid-phase system, the oxygen transfer rate can be enhanced by increasing power inputs for agitation, aeration rate, and oxygen partial pressures. Generally, increase of agitation speed leads to an improved dispersion of the gas phase, which increases the volumetric interfacial area, and the greater turbulence, which reduces the mass transfer resistance across the gas-liquid interface [65]. Increasing the aeration rate would have the same effect in the gas-liquid interfacial area. Oxygen transfer rates can also be enhanced by increasing the oxygen partial pressure in the gas phase, either by using oxygen-enriched air or by increasing the operating pressure. This leads to a higher saturation concentration of oxygen in the liquid medium. However, the use of oxygen-enriched air could be restricted by cost and safety, particularly in oxidation processes with explosive petrochemicals as substrates and/or products [20], whereas operation at pressures up to 1 to 2 bar would be possible in most bioreactors.

Substrate Transport

The transport of apolar substrates to enzymes may influence the efficiency of a reaction system. For instance, the mass transfer of lipophilic substrates from the organic to the aqueous phase limited the product yield and the kinetic resolution of racemic epoxides by an epoxide hydrolase in an octane/aqueous two-liquid phase system [7].

Apolar substances are transferred to cells via simple diffusion in an aqueous single-phase system. The transport rate of apolar substrates to intracellular enzymes can be estimated based on bioconversion rates or substrate uptake rates. The pseudocumene oxidation activity of *E. coli* JM101 (pSPZ3) reached 250 U/g CDW in an aqueous single-phase system [16]. This indicates that the aromatic compound was transported to the cells at a rate of at least 250 μmol/g CDW/min. Furthermore, the K_S of whole-cell biocatalysts for such aromatic compounds including pseudocumene, toluene, and styrene ranged from 10 to 200 μM [16, 88]. Thus, the substrate concentrations over 40 to 800 μM (4-fold of K_S) would not limit the target reactions in the order of 250 U/g CDW in aqueous single-phase systems.

Lipophilic substrates in a two-liquid-phase system (Fig. 5) have been suggested to transfer from an organic phase to cells via the aqueous phase, via mediators (*e.g.*, emulsifying agents), or through direct contacts between dispersed organic droplets and cells [40]. The transport efficiency of apolar substrates to whole-cell biocatalysts in the biphasic systems can be estimated based on biotransformation rates. The recombinant *E. coli* JM101 (pSPZ10) expressing the *styAB* of *Pseudomonas* sp. VLB120 showed 60 U/g CDW with respect to styrene epoxidation in a BEHP-based two-liquid-phase system (phase ratio of 0.5) [88]. This indicates that styrene was transported from the organic phase to the cells at a rate of over 60 μmol/g CDW/min. Furthermore, the K_S of the whole-cell biocatalysts for the aromatic compound was 14 mM in the organic phase [87]; the biotransformation rates were a function of substrate concentrations in the organic phase. Thus, the substrate concentrations in the organic phase higher than 56 mM (4-fold of K_S) would not limit the target reactions in the range of 60 U/g CDW in organic/aqueous two-liquid-phase systems.

In addition, the volumetric productivity of styrene epoxidation of the recombinant *E. coli* in the biphasic system reached 1,800 U/l_{aq} at a styrene concentration of 70 mM in the organic phase [88]. This indicates that the phase transfer rate of styrene from the organic carrier solvent to the aqueous phase increases up to 1,800 μmol/l_{aq}/min at a styrene concentration over around 70 mM in the reaction medium. Therefore, mass transport may not limit the volumetric productivity, at least in the order of 1,800 U/l_{aq} when the cell densities are around 30 g CDW/l.

OUTLOOK

The volumetric productivities of oxidative biotransformations are steadily increasing owing to deeper understanding of the factors that limit bioconversion performance of oxygenase-based biocatalysis. It is anticipated that the efficiency of whole-cell biocatalysis will be further increased via functional expression of oxygenase genes, increase of cofactor regeneration capacity, and attenuation of product toxicity. Improvement in the biocatalyst efficiency will enable the commercial production of medium-priced chemicals (*ca.* 10US$/kg) as well as chiral compounds.

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