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Whole-cell biocatalysis for selective and productive C–O functional group introduction and modification

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During the last decades, biocatalysis became of increasing importance for chemical and pharmaceutical industries. Regarding regio- and stereospecificity, enzymes have shown to be superior compared to traditional chemical synthesis approaches, especially in C–O functional group chemistry. Catalysts established on a process level are diverse and can be classified along a functional continuum starting with single-step biotransformations using isolated enzymes or microbial strains towards fermentative processes with recombinant microorganisms containing artificial synthetic pathways. The complex organization of respective enzymes combined with aspects such as cofactor dependency and low stability in isolated form often favors the use of whole cells over that of isolated enzymes. Based on an inventory of the large spectrum of biocatalytic C–O functional group chemistry, this review focuses on highlighting the potentials, limitations, and solutions offered by the application of self-regenerating microbial cells as biocatalysts. Different cellular functionalities are discussed in the light of their (possible) contribution to catalyst efficiency. The combined achievements in the areas of protein, genetic, metabolic, and reaction engineering enable the development of whole-cell biocatalysts as powerful tools in organic synthesis.

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1. Introduction

The catalytic regio-, stereo-, and chemoselective generation or alteration of C–O functionalities is a basic chemical concept in nature,

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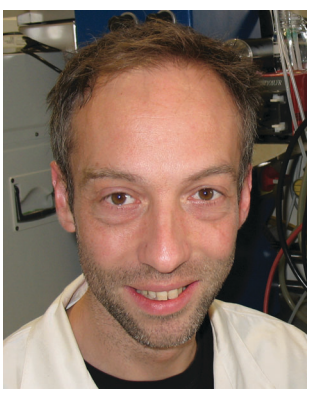

Mattijs K. Julsing

Mattijs K. Julsing studied pharmacy at the University of Groningen and graduated in 2001. During his PhD studies (2001–2006) under the supervision of Prof. Wim Quax and Prof. Oliver Kayser at the Department of Pharmaceutical Biology at the University of Groningen, he investigated bioconversion and combinatorial biosynthesis approaches for natural products. In 2007, he joined the Laboratory of Chemical Biotechnology at TU Dortmund University as a postdoctoral research associate, where he leads the research group Natural Product Biotechnology since June 2011. His research focuses on the development of biotechnological approaches for the synthesis of natural products using microbial host organisms.

mediated by enzymes. With only very few exceptions like hydratases, most of the enzymes involved are oxidoreductases typically depending on cofactors and often featuring a homo- or even heteromultimeric structure with a limited stability. In technical applications, *e.g.*, in chemical synthesis, enzyme stability is of major importance as evolution did not optimize nature's catalysts for technical process conditions. Stabilization is traditionally achieved by enzyme immobilization on or in artificial matrices,[1] especially for simple enzymes or synthesis reactions running on a small scale with respect to product amounts. In most synthetic biotechnological processes operated on large scales, enzymes are protected (immobilized) in a microbial cell.[2] So-called whole-cell biocatalysts may be applied as living microorganisms or metabolically inactive (dead) cells. In either case, the functional unit is the individual cell. Enzymes are, of course, contributing catalytic activities, yet, critical parameters for catalyzing the turnover of a substrate to a product like specificity, selectivity, productivity (space time yield), and catalytic efficiency (turnover number, total turnover number) are overall determined by cellular features. Fig. 1 schematically highlights reaction boundaries like the turnover rate, toxicity, catalyst inactivation over time (total turnover number), and downstream processing using a window of operation for whole-cell biocatalysis as introduced by Woodley and Titchener-Hooker.[3]

Biocatalysis using isolated enzymes is governed by the biochemistry of proteins and their cofactors and coenzymes. In analogy, biocatalysis using whole microbial cells is additionally controlled by mass transfer, cellular metabolism, protein synthesis, compartmentalization, and growth and inactivation of the cell. The combination of catalysis-based technical syntheses with life opens unique and powerful perspectives and possibilities for designing reactions and processes, especially for stabilizing and even regenerating the biocatalyst during a reaction. On the other hand, it implicates the challenge of working at the interface of and combining biology, chemistry, and biochemical engineering. This review article covers all issues concerning the selective and productive introduction and modification of C–O functional groups using whole-cell biocatalysis as means of stabilization. After introducing metric parameters to characterize a whole-cell biotransformation, reaction examples are presented in a sequence of complexity (called continuum, Fig. 2) to allow the reader to allocate and recognize the respective type of reaction. Basic parameters controlling the functionality of a cellular biocatalyst are discussed and highlighted in Section 4. Each reaction and biocatalyst type necessitates a specific design and handling, which is highlighted by individual reaction engineering concepts in Section 5. Stabilizing and maximizing biocatalyst activity and efficiency as well as volumetric productivity using whole cells is certainly not limited to selective C–O chemistry but applies to

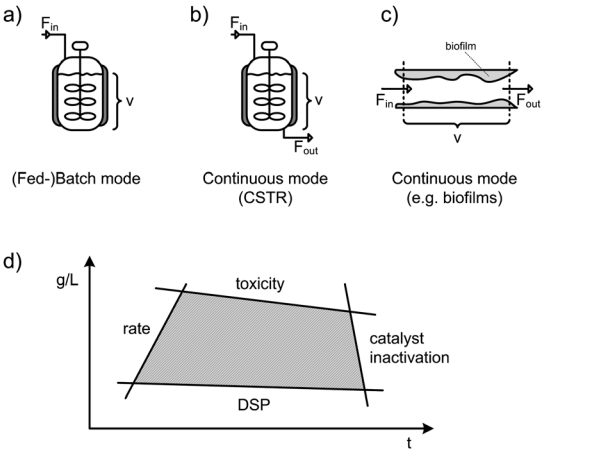

Fig. 1 Different process concepts applied in biocatalytic whole-cell reactions (a–c) and the process window which defines the boundaries of such processes. v, operating volume; CSTR, continuous stirred tank reactor; DSP, down-stream processing.

Bruno Bühler

Bruno Bühler studied biology at the Swiss Federal Institute of Technology (ETH) in Zurich with a specialization in biotechnology and graduated in 1998. He received his PhD degree in 2003 for his work on specific xylene oxyfunctionalization by whole-cell multistep biocatalysis at the Institute of Biotechnology at ETH and was awarded the ETH medal for outstanding PhD theses. After a postdoctoral period under Andreas Schmid at ETH, he joined the Laboratory of Chemical Biotechnology at TU Dortmund University in October 2004 as a leader of the Applied Biocatalysis group focusing on rational cell and process engineering for biocatalytic oxyfunctionalization reactions.

Andreas Schmid

Andreas Schmid graduated in microbiology (1992) at the University of Stuttgart, where he received his doctoral degree in 1997. From 1996–1998, he performed postdoctoral studies with Prof. Bernard Witholt at the Institute of Biotechnology, Swiss Federal Institute of Technology (ETH) in Zurich, where he became group leader in 1998 and did his habilitation in 2004 (venia legendi for Microbiology and Biotechnology). Since October 2004, he has been Professor for biotechnology and has been heading the Laboratory of Chemical Biotechnology at TU Dortmund University. His research areas include single cell analysis, nano- and microreactors, catalytic biofilms, sustainable redox biocatalysis, and systems biotechnology.

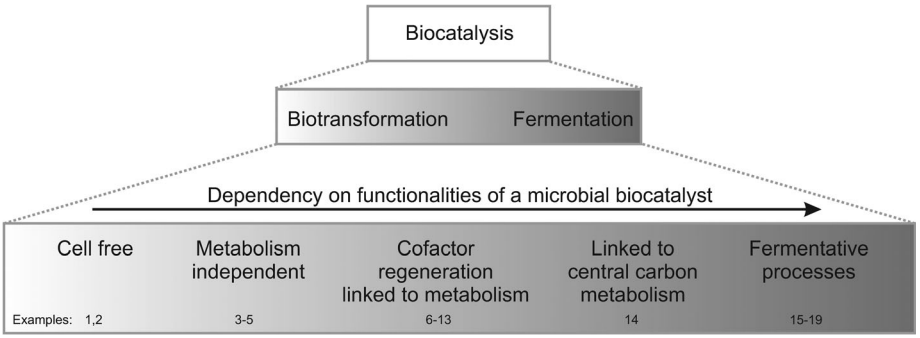

Fig. 2 Biocatalysis as a continuum. The numbers from 1–19 refer to the entries in Table 2.

all complex, but also simple, enzyme systems used to synthesize materials, chemicals, or even energy carriers of the future.

2. Key parameters in biocatalysis

The characterization of biocatalysts and respective reactions and processes requires the use of defined parameters. Biochemical publications often use relative numbers such as conversion and isolated yields or relative activities. For a direct quantitative evaluation and comparison, however, quantitative uniform parameters are required (and should be used).[4] Such quantitative parameters include specific biocatalyst activity and kinetics, yield on biocatalyst (TTN), volumetric productivity, and the product concentration achieved.[5,6] Noteworthily, parameters of whole-cell approaches (may) differ slightly from their equivalents in cell-free processes (Table 1). Typically, in protein biochemistry and enzyme application in organic chemistry, bioconversion rates are given as turnover number (TN) or turnover frequency (TF) in $mol_{product}\ mol_{enzyme}^{-1}\ s^{-1}$ or, alternatively, as reaction velocity in $mol_{product}\ g_{enzyme}^{-1}\ s^{-1}$ or specific activity (U g_{enzyme}^{-1}), where one unit (U) is typically defined as one μmol product formed per minute. In whole-cell biocatalysis, specific product formation rates are usually calculated relative to the amount of biomass (cell dry weight: CDW). Specific activities are then calculated as U g_{CDW}^{-1}. Yields on biocatalyst for cell-free enzymatic bioconversions are given as the total turnover number (TTN; $mol_{product}\ mol_{enzyme}^{-1}$) as in chemical catalysis, whereas this parameter is given as $Y_{p/x}$ in $g_{product}\ g_{CDW}^{-1}$ for whole-cell processes. Finally, enzyme kinetics are parameterized using the Michaelis dissociation constant K_m, the maximal turnover frequency k_{cat}, and the specificity constant k_{cat}/K_m. In whole-cell biocatalysis, the equivalents are the uptake constant K_s, as it is used in the Monod kinetics of microbial growth, the maximum reaction velocity V_{max}, and the specificity constant V_{max}/K_s. Both K_m and K_s are expressed as the substrate concentration, at which the half maximal specific activity is reached. In addition to the parameters mentioned, metabolic fluxes, typically given in mol $g_{CDW}^{-1}\ h^{-1}$, are crucial parameters for whole-cell processes to describe reaction rates in intracellular metabolic pathways as well as external rates, *i.e.*, substrate uptake and product formation rates.

In order to evaluate the performance of bioprocesses and to identify the limiting parameters, the operational window concept has been introduced[3] as discussed in the Introduction (Fig. 1). This concept was applied to determine the feasibility of bioprocesses by setting minima for duration (h), volumetric productivity (space time yield in g $L^{-1}\ h^{-1}$), and product concentration (g L^{-1}).[7–10] However, other parameters can also be compared in operational windows.[3]

3. The reaction spectrum of biocatalytic C–O functional group chemistry – microbial cells as bioreactors

Biocatalysis offers highly regio- and stereoselective alternatives to traditional chemical syntheses[2,11,12] with the selective oxyfunctionalization of unactivated carbon atoms being of particularly high synthetic value.[13–17] Whereas lyase-catalyzed

Table 1 Key parameters for the characterization and quantification of cell-free and whole-cell processes

	Cell-free processes		Whole-cell processes	
	Parameter	Unit	Parameter	Unit
Rate	Specific activity	U g_{enzyme}^{-1}	Specific activity	U g_{CDW}^{-1}
	Turnover number (TN) Turnover frequency (TF)	$mol_{product}\ mol_{enzyme}^{-1}\ s^{-1}$	Metabolic flux	mol $g_{CDW}^{-1}\ h^{-1}$
Yield	Product yield on substrate ($Y_{p/s}$)	$mol_{product}\ mol_{substrate}^{-1}$	Product yield on substrate ($Y_{p/s}$)	$mol_{product}\ mol_{substrate}^{-1}$
	Total turnover number (TTN)	$mol_{product}\ mol_{enzyme}^{-1}$	Product yield on catalyst ($Y_{p/x}$)	$g_{product}\ g_{CDW}^{-1}$
Productivity	Space time yield (STY)	$g_{product}\ L^{-1}\ h^{-1}$	Space time yield (STY)	$g_{product}\ L^{-1}\ h^{-1}$
Affinity	K_m	$mol_{substrate}\ L^{-1}$	K_s	$mol_{substrate}\ L^{-1}$
Catalyst efficiency	k_{cat}/K_m	$M^{-1}\ s^{-1}$	V_{max}/K_s	U $g_{CDW}^{-1}\ M^{-1}$

U = $\mu mol_{product}\ min^{-1}$; CDW, cell dry weight.

oxyfunctionalization is restricted to double bond hydration, oxidoreductases can be considered to be highly versatile, representing the most prominent enzyme class capable of performing C–O functional group chemistry. Oxidoreductases often consist of multiple protein components, have a low stability in isolated form, and are redox-cofactor dependent. These factors favor their application in a protected, self-renewing, cofactor regenerating, and reactive oxygen species degrading environment: living cells.[11,13,17–20] Biocatalytic processes are typically divided into two categories: (i) biotransformations and (ii) fermentations. Biotransformation reactions can be catalyzed by either isolated enzymes or whole cells. Generally, cheap organic carbon like *e.g.*, glucose, glycerol, or an organic acid is applied for biocatalyst production and may additionally serve as a source of energy and reduction equivalents for cell maintenance and cofactor regeneration during biotransformations, where an additional substrate is transformed into a value-added product by single- or multistep catalysis. In classical fermentations instead, the synthesis of the target product by whole cells is achieved directly from the added carbon and energy source. In reality, this rough division in only two categories can be profiled as a continuum, ranging from *in vitro* biocatalysis using isolated enzymes to whole-cell-based biotransformations uncoupled from metabolism down to metabolism-coupled biotransformations and finally to classical fermentations and fermentations involving pathway engineering (Fig. 2). In this continuum, concepts for biocatalyst, reaction, and process engineering overlap and also depend on the type of substrates and products handled. In the following, we will further define such a continuum and allocate reaction examples involving biocatalytic C–O chemistry to this transition from cell-free biocatalysis to fermentation with special emphasis on advantages and possible limitations of using whole cells as enzyme immobilizing compartments. Thereby, this large section aims at highlighting the large diversity of enzymes performing equally diverse C–O functional group chemistry and the differing type and degree of dependency of catalytic efficiency on microbial physiology. The respective enzymes considered here include oxidoreductases such as dehydrogenases, oxygenases, oxidases, and peroxidases as well as hydratases catalyzing the hydration of double bonds in a lyase-type of reaction. Dependencies on cell physiology include enzyme synthesis and regeneration, substrate accessibility *via* membranes, redox cofactor regeneration, deactivation of reactive oxygen species, stress responses such as solvent tolerance, energy supply, and provision of cosubstrates and/or building blocks for product synthesis *via* the cell metabolism.

3.1 Oxyfunctionalization reactions using cell-free systems

Monooxygenase catalysis for epoxidations in cell-free reactions. The *in vitro* application of styrene monooxygenase (StyAB) for the asymmetric epoxidation of different vinyl aromatics to their enantiopure epoxides is a good example for cell-free monooxygenase catalysis, tackling common hurdles of such approaches (Table 2, entry 1).[21,49] The two-component enzyme system

Table 2 Catalysts and reactions reviewed to show the broad spectrum of biocatalytic C–O functional group chemistry

Entry	Catalyst	Reaction	Ref.
Oxyfunctionalization reactions using cell-free system			
1	Styrene monooxygenase	Styrene → (*S*)-styrene oxide	21
2	Chloroperoxidase	Indole → 2-oxoindole	22, 23
Selective C–O chemistry based on whole-cell biotransformations			
Metabolism-independent catalysis based on whole cells			
3	Fumarate hydratase	Fumaric acid → malic acid	24
4	Alcohol dehydrogenase	Asymmetric reduction of ketones	25
5	Toluene dioxygenase and dihydrogenase	Toluene → toluene dihydrodiol → 3-methylcatechol	26, 27
$NAD(P)^+$-regeneration by cellular metabolism			
6	Dehydrogenase	Nicotinic acid → 6-hydroxynicotinic acid	28, 29
NAD(P)H regeneration via glucose catabolism			
7	Alcohol dehydrogenase	2,5-Hexanedione → (2*R*,5*R*)-hexanediol	30, 31
8	CYP	POPS → HPOPS	32, 33
9	Cyclohexanone monooxygenase	Baeyer–Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one	34, 35
10	Styrene monooxygenase	Styrene → (*S*)-styrene oxide	36, 37
Multistep oxidation depending on cell metabolism			
11	CYP450/oxidases/dehydrogenases	Alkanes → dicarboxylic acids	38, 39
12	Xylene monooxygenase	Pseudocumene → 3,4-dimethylbenzaldehyde	40, 41
13	Desaturase/hydratase	4-Butyrobetaine → L-carnitine	29
Oxygenation with a direct link to central carbon metabolism			
14	Proline-4-hydroxylase	Proline → 4-hydroxyproline	42
Fermentative processes involving C–O functional group chemistry			
Fermentative production of bulk and fine chemicals			
15	Amino acid biosynthesis/proline-4-hydroxylase	Glucose → 4-hydroxyproline	43
16	Engineered catabolic pathway	Glucose → propane-1,3-diol	44
17	Amino acid biosynthesis/naphthalene dioxygenase	Glucose → indigo	45, 46
Fermentative production of biologically active terpenoids			
18	Engineered steroid biosynthesis pathway	Glucose → hydrocortisone	47
19	Engineered isoprenoid biosynthesis pathway	Glucose → artemisinic acid	48

CYP, cytochrome P450 monooxygenase.

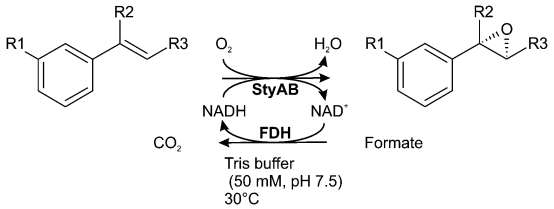

Fig. 3 *In vitro* epoxidation of vinyl aromatics by styrene monooxygenase (StyAB) with NADH regeneration by formate dehydrogenase (FDH). Substituents: R1 = H, Cl; R2 = R3 = H, CH_3.

consists of the actual oxygenase subunit (StyA) and a reductase subunit (StyB).[50] By oxidation of the redox cofactor NADH, StyB delivers the electrons necessary for oxygen activation *via* the diffusible cofactor $FADH_2$ to StyA. The dependence of StyAB on expensive cofactors necessitates the introduction of an NADH regeneration system. For this purpose, formate dehydrogenase (FDH) and formate were used for NAD^+ reduction (Fig. 3).[21] In order to prevent substrate and product inhibition, dodecane was added to the reaction mixture as a second phase, functioning as a substrate reservoir and product sink. Such an *in situ* product removal (ISPR, see Section 5) also facilitates downstream processing.[17,51,52] Different vinyl aromatics (50 mM) were converted to the corresponding epoxides with isolated yields of up to 87% and an enantiomeric excess (ee) of >98%. Denaturation of the enzyme at the organic/aqueous interface was reduced by addition of bovine serum albumin (BSA). At high aeration rates, denaturation of proteins also occurred at the gas/liquid interface. Aeration is necessary, since O_2 is a cosubstrate in the epoxidation reaction. Therefore, a trade-off between the aeration rate and enzyme stability had to be found. Addressing the mentioned challenges, gram-scale production of enantiopure epoxides was achieved with volumetric productivities ($\sim$1 g L^{-1} h^{-1}) in the same order of magnitude as whole-cell styrene epoxidation with recombinant *E. coli* containing StyAB (4.2–4.5 g L^{-1} h^{-1}).[53,54]

Chloroperoxidase-catalyzed oxyfunctionalization. Peroxidases such as heme-dependent chloroperoxidase (CPO) have also been reported to catalyze oxyfunctionalization reactions such as sulfoxidations, hydroxylations, and epoxidations. These reactions as well as halogenations and alcohol oxidations are catalyzed with high activity and enantioselectivity, using hydrogen peroxide (H_2O_2) or other organic peroxides as oxidants, relieving the dependency on redox cofactors as electron donors (Fig. 4).[22,23,55–59] Thereby, the substrates undergo cytochrome P450 monooxygenase (CYP)-like reactions.[60] High maximum total turnover numbers (TTN) of 8.6×10^5 and 2.5×10^5 were reported for the oxidation of indole to 2-oxoindole (Table 2, entry 2) and thioanisole to

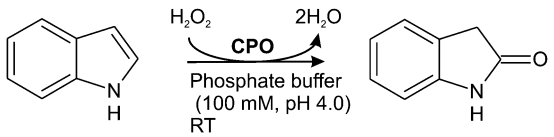

Fig. 4 Indole oxidation to 2-oxoindole as an example for an oxygen transfer reaction catalyzed by chloroperoxidase (CPO).

(*R*)-methyl phenyl sulfoxide, respectively.[22,57,61] The application of peroxidases in industry, however, remains challenging due to the low operational stability in the presence of H_2O_2, which was reported to cause enzyme deactivation *via* oxidative deterioration of the porphyrin ring in heme-dependent peroxidases (such as CPO).[22,59,62] Classical approaches to increase the stability and concomitantly the yield on catalyst (TTN) of CPOs include a stepwise or constant H_2O_2 feed[63,64] or H_2O_2 sensors and dosing systems for an oxidant feed-on-demand.[65] A fascinating approach to minimize the H_2O_2-induced inactivation of CPOs is the *in situ* generation of the oxidant *via* addition of glucose, oxygen, and glucose oxidase (GOX) to the reaction mixture.[22] By this approach, the TTN for CPO catalyzing 5-methoxyindole and *cis*-2-heptene oxidation was almost doubled. A further improvement was achieved by co-immobilization of CPO and GOX in polyurethane foam resulting in a four-fold increased TTN for indole oxidation. CPOs are able to retain their activity in low-water media, particularly in *tert*-butyl alcohol–water mixtures containing up to 70% (v/v) of *tert*-butyl alcohol functioning as a cosolvent for poorly water-soluble substrates.[66] Additionally, *tert*-butyl alcohol stabilizes CPOs, presumably by acting as a hydroxyl radical scavenger.[65] Remarkably, CPO-catalyzed reactions have also been achieved in water-free systems.[67] Despite the considerable industrial potential of CPOs, commercialization is difficult due to their high price and low operational stability.[68]

Both *in vitro* approaches introduced demonstrate the versatility and limitations of isolated oxidoreductases for the selective introduction of an oxygen atom into organic molecules. Although a lot of progress regarding redox-cofactor regeneration in cell free systems was achieved[69–73] and reaction engineering approaches have been shown to alleviate stability issues, respective processes are restricted to the small scale production of high value-added compounds. Criteria for industrial implementation to produce fine and bulk chemicals are hardly met.

3.2 Selective C–O chemistry based on whole-cell biotransformations

As mentioned above, whole cells provide a natural environment for enzymes with respective potential advantages over cell-free systems (see Section 4). Additionally, the metabolism of living cells can be exploited for the regeneration of cofactors and enzymes, offering an excellent basis for efficient and stable catalysis with enzymes immobilized in the confined intracellular space of a whole-cell biocatalyst. In the following, biocatalytic reactions catalyzed by whole cells are described focussing on the C–O functional group chemistry as a central element in their application on the industrial or laboratory scale.

Metabolism-independent catalysis based on whole cells. In a metabolism-independent biotransformation process, Amino GmbH and Tanabe Seiyaku Co., Ltd. produce L-malic acid from fumaric acid by means of hydratase-catalysis using *Corynebacterium glutamicum* or *Brevibacterium flavum* as suspended or immobilized cells, respectively (Fig. 5; Table 2, entry 3).[33] Thereby, fumarase ((*S*)-malate hydrolyase) catalyzes the stereoselective hydration of fumaric acid. The reaction underlies an equilibrium allowing approximately 85% conversion.

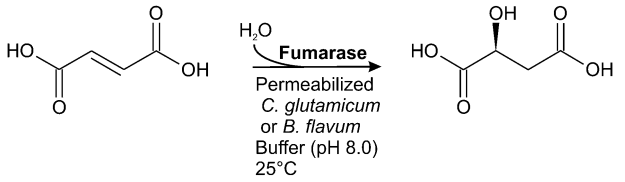

Fig. 5 Whole-cell biotransformation of fumaric acid to L-malic acid with *Corynebacterium glutamicum* or *Brevibacterium flavum*.

Fumaric acid uptake over cellular membranes is limited. Therefore, cells are typically permeabilized using detergents.[24,74,75] Such chemical treatment also reduces the formation of side products, due to the loss of cofactors necessary for these reactions (*e.g.*, malate dehydrogenase catalysis). Thus, these cells can be considered metabolically inactive. This approach based on permeabilized cells immobilizing and stabilizing the enzyme, avoiding expensive enzyme preparation and avoiding product degradation enables high yields and easy downstream processing and thus is applied for the production of several thousand tons L-malic acid per year.[33] Research also focused on fermentative malic acid production.[76–78] Due to the low carbon efficiency (molar yield of malic acid on carbon source) achieved with this approach, only the biotransformation approach using fumaric acid as the substrate is applied in industry.

The reduction of ketones to optically active alcohols by means of alcohol dehydrogenases (ADHs) depending on reduced nicotinamide cofactors (NAD(P)H) can efficiently be obtained with whole-cell biocatalysts (Table 2, entry 4).[79–82] Instead of using metabolically active cells for intracellular cofactor regeneration, the latter can be achieved by substrate-coupled or enzyme-coupled regeneration of the redox-cofactors in the cell.[83–86] In the case of substrate-coupled cofactor regeneration, the same ADH responsible for the enantioselective reduction of the ketone oxidizes a sacrificial substrate (*e.g.*, 2-propanol), thereby regenerating the cofactor. In the case of enzyme-coupled cofactor regeneration, an additional enzyme, *e.g.*, formate dehydrogenase or glucose dehydrogenase, is introduced oxidizing a sacrificial substrate (*e.g.*, formate or glucose to CO_2 or gluconolactone, respectively).[70,72] The enzyme-coupled regeneration system, applying glucose dehydrogenase together with *S*- or *R*-selective ADHs, allowed the quantitative conversion of a variety of substrates in high concentrations (>100 g L^{-1}) to the respective alcohols in high yields and with excellent ee (Fig. 6).[25] This approach was scaled up to the industrial scale at Degussa AG (now: Evonik Industries).[87] Such metabolism-independent ketone reduction impressively demonstrates the versatility of whole-cell biocatalysts for large-scale production of industrially and pharmaceutically relevant compounds.

The production of 3-methylcatechol from toluene can also be considered as a metabolism-independent biotransformation, involving a two-step oxidation catalyzed by two enzymes mutually regenerating the cofactor NAD(H) (Table 2, entry 5).[26,27,88] Toluene dioxygenase (TodCBA) and *cis*-dihydrodiol dehydrogenase (TodD) were used in recombinant strains derived from solvent-tolerant *Pseudomonas putida* F1, F107, and S12. In the case of *P. putida* F1 and F107, product degradation was avoided by using *ortho* cleavage pathway negative mutants deficient in 3-methylcatechol 2,3-dioxygenase (TodE).[26,88] The reaction sequence itself does not add a major burden to the redox-metabolism of the host cell, since every NADH molecule consumed during toluene di-hydroxylation is regenerated during the consecutive oxidation of *cis*-toluene dihydrodiol to 3-methylcatechol (Fig. 7). Therefore, this reaction can be considered "redox cofactor neutral", allowing maximal 3-methylcatechol formation rates of up to 104 U g_{CDW}^{-1}.[26] Substrate and in particular product toxicities were identified as the limiting factors and the two-liquid phase concept was applied for efficient ISPR (as reviewed in Section 5). Octanol was chosen as the solvent providing a favourable substrate and product partitioning. The use of the solvent-tolerant *P. putida* enabled the application of octanol, which is considered to be toxic for microbial cells.[89,90] To accomplish such ISPR and solvent tolerance as well as continuous enzyme synthesis, this approach required the use of living metabolically active cells. During two-liquid phase biotransformations, the high product formation rates of 104 U g_{CDW}^{-1} were not reached by far.[88] The presence of the organic phase octanol is expected to induce solvent-tolerance mechanisms, in particular energy-dependent efflux pumps. The respective physiological changes may affect the biocatalytic reactions, *e.g.*, *via* the redox cofactor balance or active substrate efflux (reviewed in Section 4.4).[91–94]

NAD(P)$^+$-regeneration by cellular metabolism. At Lonza AG, *Achromobacter xylosoxidans* is applied for the regioselective hydroxylation of nicotinic acid to 6-hydroxynicotinic acid (Fig. 8; Table 2, entry 6).[28,29,95] The $NADP^+$ dependent nicotinate dehydrogenase is the first enzyme in the bacterial degradation pathway of nicotinic acid and is responsible for heterocycle hydroxylation *via* the incorporation of oxygen derived from water with $NADP^+$ serving as electron acceptor.[96] The host metabolism assures regeneration of the cofactor, in this case in the oxidative direction. The wild-type strain applied is able to grow on

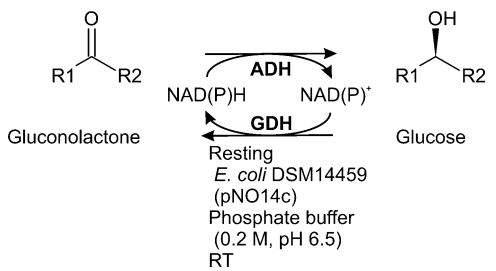

Fig. 6 Asymmetric ketone reduction independent of the cell metabolism using recombinant *E. coli* DSM 14459 cells containing the respective alcohol dehydrogenase (ADH) and glucose dehydrogenase (GDH). RT: room temperature.

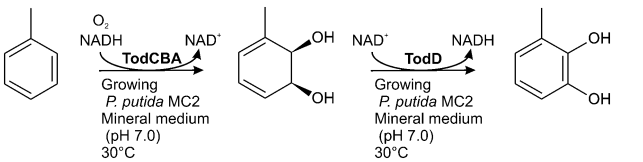

Fig. 7 "Redox-neutral"-conversion of toluene to 3-methylcatechol catalyzed by growing solvent-tolerant *P. putida* MC2 containing toluene dioxygenase (TodCBA) and *cis*-dihydrodiol dehydrogenase (TodD).

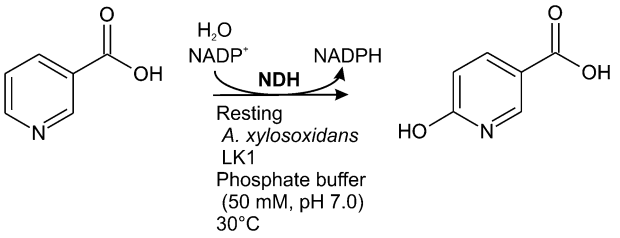

Fig. 8 Hydroxylation of nicotinic acid to 6-hydroxynicotinic acid with resting *Achromobacter xylosoxidans* cells containing nicotinate dehydrogenase (NDH).

nicotinic acid as the sole source of carbon and nitrogen. Nevertheless, accumulation of 6-hydroxynicotinic acid occurs, since the second enzyme of the pathway, *i.e.*, 6-hydroxynicotinate hydroxylase, is strongly inhibited in the presence of 1% (w/v) nicotinic acid. Substrate inhibition of nicotinate dehydrogenase does not occur. During the initial growth phase, non-inhibiting nicotinic acid concentrations enable bacterial growth. Subsequently, the biotransformation is initiated by addition of nicotinic acid in excess. This two-stage process enabled 6-hydroxynicotinic acid formation up to a titer of 74 g L^{-1} in 25 h and runs on a multi-ton scale. The product can be isolated *via* precipitation induced by a pH shift resulting in isolated yields of up to 91% with a purity of 99%.[29]

NAD(P)H regeneration *via* glucose catabolism. The asymmetric reduction of ketones and diketones by ADHs allows the formation of enantiopure alcohols and diols, respectively. *E.g.*, the ADH from *Lactobacillus kefir* catalyzing this reaction accepts a wide variety of substrates and is NADPH dependent.[97,98] For cofactor regeneration, the native glucose catabolism of living *Lactobacillus kefir* was exploited for the reduction of 2,5-hexanedione to (2*R*,5*R*)-hexanediol (Fig. 9; Table 2, entry 7).[30,31] Thereby, glucose serves as a cheap source of reducing equivalents providing up to 12 reduced cofactor equivalents (in the form of NADH, NADPH, or $FADH_2$) per molecule of glucose oxidized to CO_2.[94] These reducing equivalents are also consumed in the cellular energy metabolism contributing to the stabilization and renewal of the biocatalytically active enzymes (as discussed in Sections 4.2 and 4.3). Per mol reduced oxo-function, 0.5 mol of glucose were necessary resulting in an equimolar ratio of substrate and co-substrate necessary for the reaction. In batch reactions for the reduction of 2,5-hexanedione, the intermediate (*R*)-5-hydroxyhexan-2-one initially accumulated before conversion to the desired product is achieved, indicating different reaction rates for each step.[31] By applying a substrate and co-substrate feed in a molar ratio of 1 : 1 and by fine-tuning of the feed rate, the selectivity ($c_{product}(c_{product} + c_{intermediate})^{-1}$) towards (2*R*,5*R*)-hexanediol was significantly increased. Additionally, 100% substrate conversion was achieved using the right feeding strategy. In order to improve the reduction reaction further, a continuous production process was developed.[30] The authors identified the yield of product on applied biomass ($g_{product}\ g_{biomass}^{-1}$) as the key parameter for an economically feasible process. The continuous reaction setup allowed a 30 and 3.3-fold yield improvement ($g_{product}\ g_{biomass}^{-1}$) compared to batch and fed-batch processes, respectively. Again, almost quantitative conversion was achieved with a space time yield (STY) of 64 g L^{-1} d^{-1} over 5 days. Although significant amounts of the intermediate (*R*)-5-hydroxyhexan-2-one were present throughout the entire process, the achieved final (2*R*,5*R*)-hexanediol conversion yield of 78% was sufficient for an efficient downstream processing based on crystallization (requirement: >60% selectivity).

At BASF, the ascomycete *Beauveria bassina* is used for the production of (*R*)-2-(4-hydroxyphenoxy) propionic acid (HPOPS) from (*R*)-2-phenoxypropionic acid (POPS) (Fig. 10; Table 2, entry 8)[32] *via* CYP-catalyzed regioselective aromatic hydroxylation.[99] Two rounds of random mutagenesis generated a mutant strain (Lu 700) able to produce 7 g L^{-1} d^{-1} of HPOPS, representing a 23-times higher STY as compared to the wild-type strain (0.3 g L^{-1} d^{-1}). Besides substrate tolerance and productivity as selection criteria, mutants were selected based on their morphology, *i.e.*, growth in yeast-like fashion rather than producing mycelia, since sufficient aeration of a mycelium forming culture is difficult. Furthermore, the trace element composition was improved based on a genetic algorithm,[100] and a two-stage process (short growth phase and prolonged production phase) was established in order to improve the yield of HPOPS on glucose. The entity of these refinements allowed a scale-up from lab-scale to 100 m^3 maintaining the same productivity. Whole cells also proved to be suitable catalysts for the production of other hydroxylated aromatic carboxylic acids.

The oxygenation of linear and cyclic ketones to esters or lactones, respectively, can be catalyzed highly enantio- and regioselectively by Baeyer–Villiger monooxygenases (BVMOs).[101–104] BVMOs use molecular oxygen and the redox-cofactor NAD(P)H as the reductant for the Baeyer–Villiger type oxidation.[105,106] The best characterized BVMO is the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*.[34] This enzyme is used for the regiodivergent production of a 1 : 1 mixture of the lactones (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one

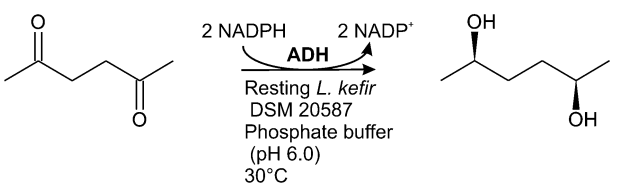

Fig. 9 Asymmetric reduction of 2,5-hexanedione to (2*R*,5*R*)-hexanediol with wild-type *Lactobacillus kefir* resting cells containing an alcohol dehydrogenase (ADH).

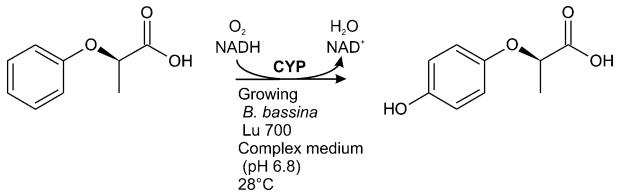

Fig. 10 Hydroxylation of (*R*)-2-phenoxypropionic acid (POPS) to (*R*)-2-(4-hydroxyphenoxy) propionic acid (HPOPS) *via* cytochrome P450 monooxygenase (CYP) catalysis in growing *Beauveria bassina* Lu 700.

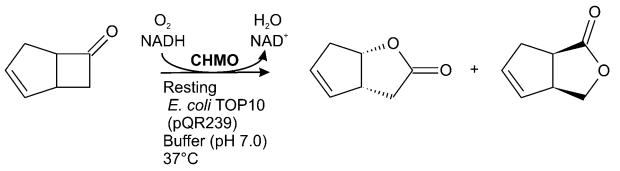

Fig. 11 Regiodivergent oxidation of bicyclo[3.2.0]hept-2-en-6-one to (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one catalyzed by cyclohexanone monooxygenase (CHMO) containing recombinant *E. coli* TOP10 cells.

and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one from racemic bicyclo[3.2.0]hept-2-en-6-one in a scalable, whole-cell based reaction (Fig. 11; Table 2, entry 9).[35,107–110] Despite approaches for the *in vitro* use of CHMO with *in situ* cofactor regeneration,[111,112] application of recombinant whole cells is considered to be the most promising way for a large scale application.[109] Although the bacterial metabolism efficiently regenerates the redox-cofactor, the whole-cell biotransformation faces some obstacles: (i) substrate and product inhibition (see Section 5), (ii) sufficient oxygen supply (see Section 5), and (iii) substrate uptake limitation (see Section 4.1). The first can be circumvented by substrate feeding and ISPR. A resin-based *in situ* substrate feeding and product removal (SFPR) process was developed, where an adsorption resin (Dowex Optipore L-493) enabled an increase of substrate load (from 1 g L^{-1} to 20 g L^{-1}), and consequently a higher product concentration.[113] The use of a sintered-metal sparger was applied for a scale-up to 50 L reaction volume (including the resin), addressing the second obstacle. By combining *in situ* SFPR and optimized aeration, the production of 866 g of the enantiopure lactones was achieved after only 20 h, representing a STY of 1.02 g L^{-1} h^{-1}.[110] The last obstacle, substrate uptake limitation, could not yet be solved. Whereas whole cells showed a specific activity of 55 U $g_{CDW}{}^{-1}$, 90 U $g_{CDW}{}^{-1}$ were obtained under identical conditions after cell lysis *via* sonication.

The *in vitro* application of the two-component monooxygenase StyAB for enantioselective epoxidation of vinyl aromatics has been already discussed above. As mentioned, productivities and product concentrations achieved *in vitro* were lower than those achieved in whole-cell applications, in which glucose catabolism ensured cofactor regeneration for the epoxidation of styrene to (*S*)-styrene oxide by StyAB (Fig. 12; Table 2, entry 10). Due to low aqueous substrate solubility and severe substrate and product toxicities, the two-liquid phase concept was applied using bis(2-ethylhexyl)phthalate (BEHP) as an organic carrier solvent.[36,37,53,114] BEHP is non-toxic to the cells and provided the desired extraction properties to maintain the aqueous substrate and product concentrations at sub-toxic levels. Applying this strategy, pilot-scale production of (*S*)-styrene oxide was accomplished, resulting in 307 g of isolated product with high purity (97%) after non-optimized downstream processing.[114] Interestingly, resting (*i.e.* non-growing but metabolically active) cells showed two-fold higher styrene epoxidation activities than growing cells in a comparable setup, indicating a competition of growth and biocatalysis for energy in growing cells (see Sections 4.3 and 5 for a more detailed discussion of physiological issues and reaction setups for this biotransformation, respectively).[37,115] Similar effects are also reported for the production of (+)-*cis*-(1*R*,2*S*)-1,2-naphthalene dihydrodiol by recombinant *E. coli* containing naphthalene dioxygenase.[116] The styrene epoxidation activity of resting cells, however, was less stable, which was due to a more prominent product inhibition as compared to growing cells. In general, the use of resting cells for efficient redox-biocatalysis should be considered if the stability of the catalyst allows such an application.

Multistep reactions depending on cell metabolism. Different strains of the genus *Candida* are natural producers of α,ω-dicarboxylic acids when grown on saturated or unsaturated long-chain alkanes, fatty acids, or fatty acid methyl esters (Fig. 13; Table 2, entry 11).[117–121] A two-component monooxygenase system, consisting of the catalytically active CYP52A1 and its redox-partner cytochrome P450 reductase, catalyzes the terminal hydroxylation of the substrates.[122–124] Further oxidations to yield the carboxy function are catalyzed by fatty alcohol oxidases and fatty aldehyde dehydrogenases. In unmodified strains, a large portion of the formed dicarboxylic acids undergoes β-oxidation. This leads to product degradation and chain modification, resulting in mixtures of products with different chain lengths.[125,126] By deleting the genes encoding for the acyl-CoA oxidase isozymes POX4 and POX5, yields as well as productivities were significantly improved for conversions of dodecane to dodecanedioic acid and methyl myristate to tetradecanedioic acid, supplying glucose as the co-substrate. Chain modification was not observed anymore, resulting in the exclusive formation of respective dioic acids.[38,127] The initial hydroxylation step catalyzed by CYP52A1 was identified as the rate limiting step.[128–130] Additionally, an analogous enzyme, CYP52A3, from *Candida maltosa* was shown to efficiently catalyze all the steps from the alkane to the α,ω-dicarboxylic acid.[131] Further productivity

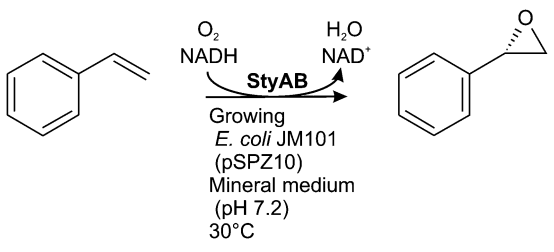

Fig. 12 Epoxidation of styrene to (*S*)-styrene oxide by growing recombinant *E. coli* JM101 containing styrene monooxygenase StyAB.

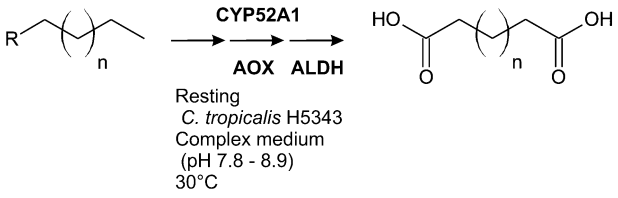

Fig. 13 Production of α,ω-dicarboxylic acids from alkanes, fatty acids, and fatty acid methyl esters by *Candida tropicalis* cells. CYP52A1 catalyzes the initial hydroxylation of the substrate. The subsequent oxidations are catalyzed by a fatty alcohol oxidase (AOX) and a fatty aldehyde dehydrogenase (ALDH). Alternatively, cytochrome P450 monooxygenases (CYPs) catalyze all oxidations. R = CH_3, COOH, $COOCH_3$; n = 8–18.

improvement was achieved by integration of additional copies of genes encoding CYP52A1 and its redox-partner into the yeast genome.[38] After engineering of the host strain, long chain α,ω-dicarboxylic acid titers of 100–200 g L^{-1} were achieved depending on the substrate. This ultimately enabled process implementation on an industrial scale at Cognis/Henkel.[132,133] Such stable conversions and consequently high titers were only achieved due to mechanisms present in intact cells coping with reactive oxygen species (ROS) (*e.g.*, catalases and superoxide dismutases) (as reviewed in Section 4.4). CYP-catalysis is typically biased by uncoupling of NAD(P)H oxidation and product formation, resulting in ROS formation.[8,134] Oxidase-catalysis also leads to the formation of ROS, typically in the form of H_2O_2. In yeast, degradation of fatty acids *via* the β-oxidation cycle, but not oxidase-catalysis related to diacid production, takes place in special compartments, the peroxisomes.[135] This compartmentalization protects the cell from ROS-induced damage and allows efficient break down of ROS (reviewed in Section 4.1).

In an effort to use a single enzyme for multistep oxidation, the xylene monooxygenase XylMA from *P. putida* mt-2 was applied in whole cells of recombinant *E. coli* and characterized as an enzyme catalyzing the multistep oxygenation of toluene and xylenes to corresponding alcohols, aldehydes, and acids (Fig. 14; Table 2, entry 12).[40,136] The use of the two-liquid phase concept allowed the exploitation of this kinetically controlled multistep reaction for the production of 22.2 and 18.4 g L^{-1} 3,4-dimethylbenzaldehyde from pseudocumene with a molar yield of 66 and 77% and a productivity of 1.7 and 1.3 g L^{-1} h^{-1} on a laboratory (2 L) and a technical (30 L) scale, respectively, enabling an isolated yield of 65% (469 g) in the latter case.[41,137] For this biotransformation, process modeling and simulation indicated an intracellular energy (NADH, ATP) shortage.[138]

The biotechnological synthesis of L-carnitine from 4-butyrobetaine at Lonza AG is a good and early example for the use of multiple steps from a native catabolic pathway implemented at the industrial scale (Fig. 15; Table 2, entry 13).[29] The soil isolate *Agrobacterium* sp. HK4 is able to grow aerobically on 4-butyrobetaine, crotonobetaine, or L-carnitine as sole carbon, nitrogen, and energy sources. Uptake of 4-butyrobetaine from the medium into the cells occurs *via* active ATP-dependent transport (see Section 4.1) and in concert with enzymatical coupling to coenzyme A (CoA). Subsequently, a double bond is introduced by a dehydrogenase reaction resulting in crotonobetainyl-CoA. Hydratase-catalyzed hydration and subsequent hydrolysis by a thioesterase finally results in the formation of L-carnitine.

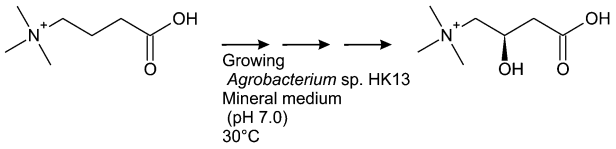

Fig. 15 Multistep L-carnitine production by growing cells of *Agrobacterium* sp. HK13.

Degradation of L-carnitine is initiated by NAD^+-dependent dehydrogenation, followed by further metabolic steps connected to the central carbon metabolism. The mutant strain *Agrobacterium* sp. HK13 lacks L-carnitine dehydrogenation activity and is therefore not able to degrade this compound. Supplying *Agrobacterium* sp. HK13 with 4-butyrobetaine as the substrate, thus, results in L-carnitine accumulation and secretion. The biotechnological synthesis from 4-butyrobetaine is more cost-efficient than chemical synthesis. Lonza started L-carnitine production using *Agrobacterium* sp. HK13 in 1993 at a 50 000 L-scale with a productivity of more than 5.4 g L^{-1} h^{-1}.[33]

In general, multi-step catalysis in the concentrated intracellular environment of microorganisms is expected to have high potential and impact in the field of industrial biocatalysis.[139,140]

Oxygenation with a direct link to central carbon metabolism. Utilization of cell metabolism for cosubstrate supply is not restricted to redox cofactors only. For the biotransformation of L-proline to *trans*-4-hyroxy-L-proline, a hydroxylase gene from *Dactylosporangium* sp. strain RH1 was expressed in *E. coli* (Fig. 16; Table 2, entry 14).[42] The responsible enzyme proline-4-hydroxylase is a 2-oxoglutarate dependent oxygenase and can be considered as an intermolecular dioxygenase simultaneously catalyzing the oxygenation of two substrates, namely the hydroxylation of L-proline to *trans*-4-hyroxy-L-proline and the oxidative decarboxylation of 2-oxoglutarate to succinate and CO_2. Thereby, all electrons required for O_2 reduction come from the two substrates. The cosubstrate and electron donor 2-oxoglutarate is an intermediate of the citric acid cycle and was thus derived from glucose *via* the central carbon metabolism of living *E. coli* cells. With succinate as the coproduct, proline hydroxylase creates a shortcut in the citric acid cycle (see Section 4.3 for an overview on metabolism-related aspects). To promote L-proline hydroxylation in *E. coli*, endogenous substrate degradation was blocked by a deletion of the gene encoding the first enzyme (PutA) in the L-proline degradation pathway. For the bioconversion of L-proline into *trans*-4-hydroxy-L-proline, the *putA* knockout of *E. coli* enabled an increase in yield from 87% to 100%.

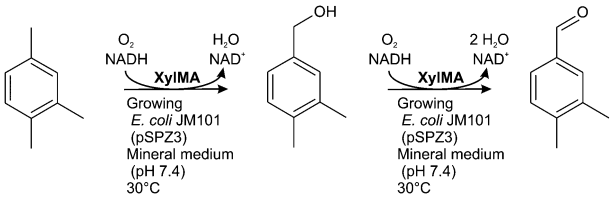

Fig. 14 Multistep oxidation of pseudocumene to 3,4-dimythylbenzaldehyde by growing recombinant *E. coli* JM101 containing xylene monooxygenase XylMA.

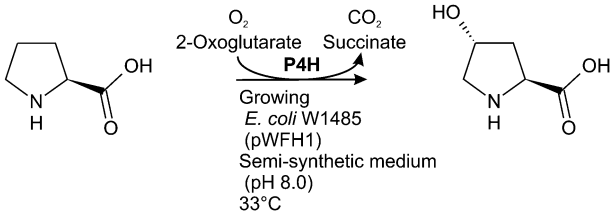

Fig. 16 Biotransformation of L-proline to *trans*-4-hydroxy-L-proline by growing recombinant *E. coli* W1485 containing proline-4-hydroxylase (P4H).

trans-4-Hydroxy-L-proline was accumulating at a STY of 0.41 g L^{-1} h^{-1} to a final product concentration of 41 g L^{-1}.[42] In this process, recombinant *E. coli* cells not only serve as a reaction compartment for the biotransformation, but also supply the cosubstrate directly *via* central carbon metabolism.

3.3 Fermentative processes involving C–O functional group chemistry

Next to biotransformations depending or not depending on host cell metabolism, endogenous catabolic and anabolic pathways of cells consisting of multiple enzymatic steps can be exploited for whole-cell biocatalysis based on cheap and renewable substrates such as glucose, glycerol, or xylose. In fact, such fermentation processes can be regarded as the use of microbial cells for multistep synthesis in one-pot with enzymes in the confined interior space of a cell. The use of metabolic pathways in microorganisms by fermentation processes for the production of ethanol, acetone, and amino acids from cheap renewable substrates is well established. The engineering of such pathways increases productivities and enlarges the spectrum of value-added compounds being synthesized. Such processes often involve sophisticated C–O functional group chemistry, in which an increasing interest from industries can be observed.

Fermentative production of bulk and fine chemicals. Based on the *E. coli* strain constructed for proline hydroxylation as described above, further metabolic engineering aimed at the use of the endogenous L-proline synthesis pathway for the synthesis of *trans*-4-hydroxy-L-proline from glucose (Fig. 17; Table 2, entry 15). In order to achieve efficient intracellular L-proline supply, two genes encoding γ-glutamylphosphate reductase (*proA*) and γ-glutamyl kinase (*proB*) were overexpressed under heterologous, non-feedback controlled, regulation. Thereby, the production of *trans*-4-hydroxy-L-proline from glucose without external L-proline addition was achieved.[43] This strain was shown to produce 25 g L^{-1} *trans*-4-hydroxy-L-proline in 96 h (0.26 g L^{-1} h^{-1}) by fermentation at a 5 L scale. The fermentation, however, suffers from low carbon efficiency (molar product yield on carbon source), and thus only the biotransformation is applied for large-scale production by Kyowa Hakko Kogyo Co. Ltd, Japan.

For bulk and fine chemicals, space time yields of at least 1.0 g L^{-1} h^{-1} have been proposed to be required to meet economic feasibility demands, whereas the minimal requirement for pharmaceuticals is considered to be 0.1 g L^{-1} h^{-1} (ref. 8 and 141) (see also Section 5). Propane-1,3-diol is a polymer synthon produced by a biotechnological fermentation process at bulk chemical scale. Many bacteria, including *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, *and Lactobacillus* species, are known to grow anaerobically on glycerol producing propane-1,3-diol in order to balance the intracellular redox state ($NADH/NAD^+$ homeostasis, see Section 4.3).[44] During such fermentation, glycerol is converted to propane-1,3-diol in two enzymatic steps, *i.e.*, dehydration followed by NADH-dependent ketone reduction. The slow anaerobic growth on glycerol limiting propane-1,3-diol titers and productivities prompted DuPont and Genencor, in a collaboration, to develop a more economically feasible process. A recombinant biosynthetic pathway was established in *E. coli* enabling the production of propane-1,3-diol in an aerobic fermentation process starting from glucose (Fig. 18; Table 2, entry 16).[44] Genes encoding glycerol 3-phosphate dehydrogenase and glycerol 3-phosphate phosphatase from *S. cerevisiae* were introduced into the *E. coli* catalyst enhancing glucose derived glycerol availability in the engineered host cell. The glycerol dehydratase complex gene *dhaB1-3* and its reactivating factors *dhaBX* and *orfX* from *Klebsiella pneumoniae* enable conversion to 3-hydroxypropionaldehyde. Finally, the NADPH-dependent YghD, a highly efficient *E. coli* homologue of the formerly used NADH-dependent *K. pneumoniae* dehydrogenase DhaT, catalyses the ketoreduction of the aldehyde to propane-1,3-diol.[44] After further modifications of the glycolysis pathway, a productivity of 3.5 g L^{-1} h^{-1} was achieved enabling a product titer of 135 g L^{-1}, a considerable improvement compared to the 3.0 g L^{-1} h^{-1} and 78 g L^{-1} obtained by anaerobic fermentation of glycerol with comparable weight yields on carbon source of 51% and 55%, respectively.[142] This process was commercialized by DuPont in collaboration with Tate & Lyle.

The *E. coli*-based production of indigo from glucose is a striking example showing how engineering of biosynthetic pathways can result in the synthesis of a fine chemical *via* the use of recombinant cells. Several microbial oxygenases (*e.g.*, naphthalene dioxygenase,[143] 2-hydroxybiphenyl 3-monooxygenase,[144] and styrene monooxygenase[145]) were found to catalyse the bioconversion of indole to indoxyl, of which two molecules spontaneously react to form indigo by non-enzymatic oxidation in air. Heterologous expression of the genes encoding naphthalene dioxygenase and the corresponding redox partners

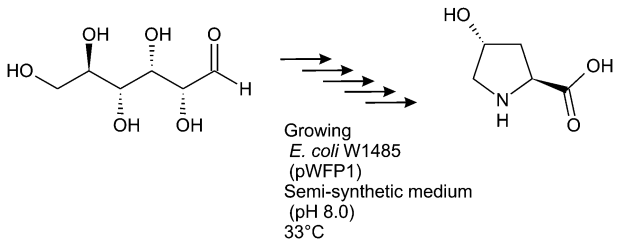

Fig. 17 Fermentative production of *trans*-4-hydroxy-L-proline from D-glucose by engineered *E. coli* W1485. The number of arrows does not represent the number of reaction steps necessary for the synthesis.

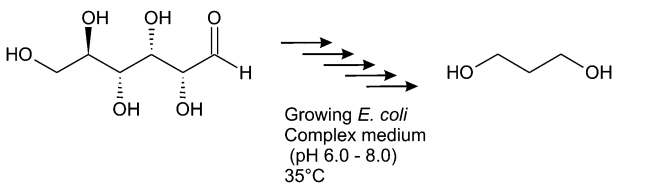

Fig. 18 Fermentative production of propane-1,3-diol from D-glucose by engineered *E. coli* cells. The number of arrows does not represent the number of reaction steps necessary for the synthesis.

ferredoxin and ferredoxin reductase from *P. putida* PpG7 in *E. coli* resulted in the formation of indigo from indole.[143] Overexpression of the tryptophanase of *E. coli* enabled biotransformation of tryptophan to indole and subsequently to indigo. As tryptophan and indole are too expensive to be used as substrates for indigo production, the synthesis of indigo from glucose was the goal of an extensive metabolic engineering effort initiated at Genencor. As a first step, microbial indole conversion into tryptophan was avoided *via* knock-out of the *trpB* gene encoding the β-subunit of the tryptophan synthase, which catalyzes the condensation of serine and indole to synthesize tryptophan. Accumulation of indole in this strain resulted in indigo production up to a titer of 135 mg L^{-1} from glucose.[45] Further improvement was achieved by overexpression of six genes involved in indole biosynthesis, increasing the intracellular availability of the indole precursor erythrose 4-phosphate *via* overexpression of the transketolase gene *tktA*,[146] and knock-out of two genes, *pykA* and *pykF*, encoding pyruvate kinases involved in the synthesis of pyruvate from phosphoenol pyruvate, another indole precursor. The obtained recombinant strain enabled Genencor to synthesize more than 18 g L^{-1} indigo from glucose in a 72 h fed-batch fermentation (Fig. 19, Table 2, entry 17).[46]

Fermentative production of biologically active terpenoids. The total biosynthesis of hydrocortisone in yeast cells is one of the first examples for fermentative steroid synthesis involving the coupling of several enzymatic steps to the endogenous steroid synthesis pathway (Fig. 20; Table 2, entry 18).[47] Hydrocortisone is an adrenal glucocorticoid of mammals and serves as a building block for the synthesis of steroidal drugs. An engineered *S. cerevisiae* strain was shown to produce up to 20 mg L^{-1} of different steroids with hydrocortisone as the main product. Despite the low productivities obtained, this approach demonstrates the versatility of microbial catalysts for high-value added drug synthesis. A combination of the recombinant expression of genes encoding multi-component and membrane associated CYPs, up-regulation of the endogenous synthesis of steroids derived from ergosterol, deletion of side reactions, and heterologous gene expression from a plasmid and *via* chromosomal integration enabled the synthesis of hydrocortisone in yeast using ethanol or glucose as substrates. This process did not address the optimization of the ergosterol biosynthesis. Ergosterol functions as the starting point of steroid derivatization in yeast and is produced from the isoprenoid precursor farnesyl diphosphate.

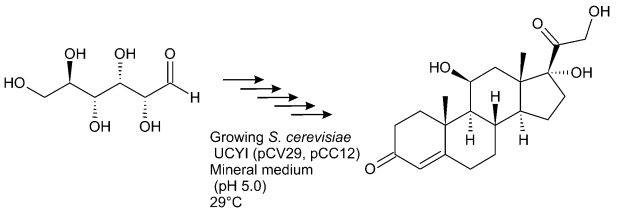

Fig. 20 Fermentative production of hydrocortisone from D-glucose by engineered *S. cerevisiae* cells. The number of arrows does not represent the number of reaction steps necessary for the synthesis.

Keasling and coworkers and Amyris Inc. did focus on the up-regulation of the isoprenoid biosynthesis pathway for the synthesis of artemisinic acid in *S. cerevisiae* (Fig. 21; Table 2, entry 19).[48,147] This terpenoid from the plant *Artemisia annua* serves as a precursor for the synthesis of the antimalarial drug artemisinin. Artemisinic acid conversion into artemisinin proceeds *via* non-enzymatic steps, both in the plant as well as *ex planta*. In yeast, the ergosterol precursor farnesyl diphosphate was converted to the sesquiterpenoid amorpha-4,11-diene by the heterologous expression of the respective plant cyclase gene. Co-expression of the genes encoding the plant enzyme CYP71AV1 and the corresponding cytochrome P450 reductase from *A. annua* enabled three consecutive oxygenation reactions transforming amorpha-4,11-diene *via* the 12-alcohol and the 12-aldehyde to artemisinic acid. Increasing the farnesyl diphosphate pool by overexpression of the genes involved in its synthesis and down-regulation of ergosterol biosynthesis by gene deletion resulted in a yeast strain producing ~100 mg L^{-1} artemisinic acid.[48] Optimization of the reaction parameters enabled a 25-fold improvement to 2.5 g L^{-1}.[148] The monooxygenase-catalysed multistep conversion of amorpha-4,11-diene to artemisinic acid appeared to be the limiting step in this process, since product concentrations up to 40 g L^{-1} have been achieved for the fermentative production of amorpha-4,11-diene.[149] The syntheses of hydrocortisone and artemisinic acid are elegant examples for the use of whole cells with engineered biosynthetic pathways including one or more oxyfunctionalizations for the synthesis of high value-added compounds. Although the productivities reached are relatively low, biotechnological production may be feasible due to the pharmaceutical interest in and the high value added to these compounds.

The processes presented in this section show that, for the application of microbial cells as biocatalysts performing

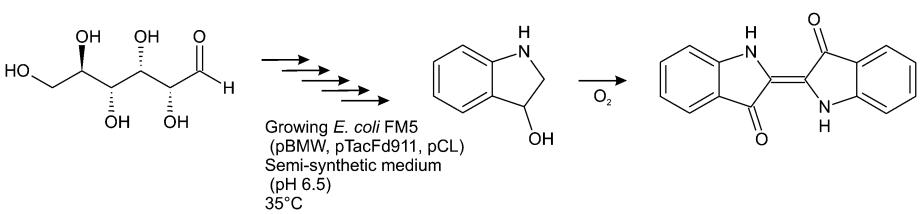

Fig. 19 Fermentative production of indoxyl from D-glucose by engineered *E. coli* cells. Indoxyl spontaneously dimerizes to indigo in the presence of molecular oxygen. The number of arrows does not represent the number of reaction steps necessary for the synthesis.

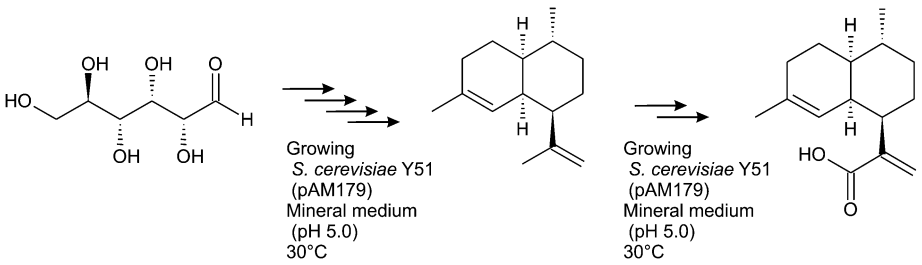

Fig. 21 Fermentative production of amorphadiene and artemisinic acid from D-glucose with engineered yeast cells. The number of arrows does not represent the number of reaction steps necessary for the synthesis.

C–O functional group chemistry, the classification in biotransformation and fermentation can be translated into a continuum, which spreads from the use of metabolically inactive cells, immobilizing an enzyme in a natural environment, to the total synthesis of high value-added natural products *via* pathways established and/or optimized by metabolic engineering. For all these processes, the cell can be regarded as a versatile catalyst unit.

4. Functionality of a cellular biocatalyst

The high potential of microbial cells for C–O functional group chemistry was proven by the success of many bioprocesses. Furthermore, possible benefits from targeted engineering strategies for these whole-cell biocatalysts were also exemplified. In order to fully exploit the synthetic potential of microbial biocatalysts, it is important to consider whole cells in their entirety as catalysts, and not only the biocatalytically active enzymes. A reduction of process intensification to the (recombinant) enzyme level only, neglecting aspects related to the cell physiology, may finally not lead to the development of a balanced, stable, productive, and efficient whole-cell workhorse. Protein engineering is extremely valuable for improving the kinetic properties or the substrate scope of an enzyme. Alone, however, it cannot address limitations related to cellular physiology, such as microbial substrate uptake, product export, enzyme synthesis, cofactor/cosubstrate supply, the energy status of the cell, toxification, and by-product formation. Therefore, the following sections of this review will define and discuss functionalities of microbial cells, which, in a concerted manner, are responsible for the extraordinary capabilities and capacities of whole-cell biocatalysts. This includes the consideration of whole-cell characteristics, which are critical with respect to whole-cell biocatalyst performance.

4.1 Membranes and mass transfer

General aspects. The use of microorganisms for productive biocatalysis defines the cell as the fundamental catalytic unit for chemical synthesis. However, whole-cell catalysts feature a much more complex structure as compared to traditional homogeneous and heterogeneous catalysts. This structure allows easy catalyst production, isolation, and recycling as well as the concerted action of multiple enzymes. Thus, such whole-cell catalysts exhibit advantageous features traditionally attained by enzyme immobilization or heterogeneous catalysis.

The outer boundary of living cells is formed by membrane structures, which separate and protect the inner environment of the cell from the outside. Cellular membranes are generally composed of phospholipid bilayers (Fig. 22).[150] In *Bacteria* and *Eukarya*, the membrane phospholipids are ester compounds of glycerol and two saturated or unsaturated fatty acid molecules with a chain length between 14 and 18 carbon atoms.[151] The third alcohol group of glycerol forms an ester with a phosphate derivative. In *Archaea*, however, the hydrophobic side chains are constituted by isoprenoids, which are connected to glycerol *via* an ether bond.[151] The bilayer, with the hydrophilic phosphate derivatives facing towards the outside and the hydrophobic hydrocarbon chains orientated to the

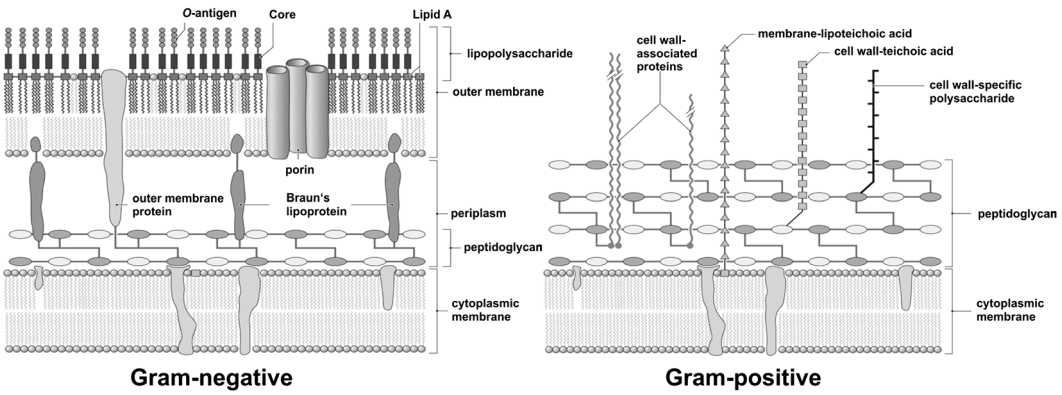

Fig. 22 Schematic overview of the organization of the cell walls of Gram-negative and Gram-positive bacteria. (Adapted from Fuchs[154])

inner side, forms a fluidic structure. Often, steroid derivatives (*e.g.* cholesterol or ergosterol in eukaryotes and hopanoids in eubacteria) are present in the bilayer to increase stability and rigidity.

Based on the differences in cell envelope structure, bacteria are divided into two groups (Fig. 22). Gram-negative bacteria, including the often applied species *E. coli* and Pseudomonads, feature not only one, but two membrane bilayers.[152] Between the cytoplasmic or inner membrane and the outer membrane is an aqueous fluidic space, the periplasm, which contains peptidoglycan. The outer membrane exhibits a layer of hydrophilic lipopolysaccharides facing towards the outside of the cell. Gram-positive bacteria such as the often applied species *Bacillus subtilis* and *Corynebacterium glutamicum* do not feature an outer membrane. Instead, the cytoplasmic membrane is covered with a peptidoglycan layer, which is much thicker and more rigid compared to that of Gram-negative bacteria.[153]

Also eukaryotic microbial cells have only one phospholipid membrane surrounding the cellular environment, but these cells do have a much more complex intracellular membrane structure. In *Eukarya* different functions are organized in specific organelles, which are separated from the cytoplasm by membranes. This compartmentalization enables cells to create suitable conditions for specific reactions.[135]

Membrane functions include the structuring and shaping of cells and organelles, protection against toxic compounds by precluding their passage, and preventing the loss of cellular components, such as metabolites, proteins, and cofactors. In whole-cell biocatalysis, membranes often play a crucial role as highlighted in the following. Membrane structures may negatively influence the performance of a whole-cell biocatalyst by (i) limiting substrate and product mass transfer,[155] (ii) effects of biocatalytic reactions on membrane-related functionalities of microbial cells, the electron transport chain being a prominent example, and (iii) effects of substrates and/or products on membrane integrity.[53,90] However, in whole-cell biocatalysis, membranes can be utilized to control substrate availability and reaction kinetics and to offer a natural stabilizing environment for enzymes, especially when membrane-bound or membrane-associated proteins are involved.

Membrane proteins as biocatalysts. Proteins are important constituents of cellular membranes fulfilling a variety of physiological and structural functions. For the biocatalytic application of membrane proteins, whole-cell systems are preferred, since these proteins can then be used in a natural environment, the membrane, which often is a prerequisite to obtain a functional protein. However, the incorporation of a functional recombinant enzyme into the membrane of a host organism is not always straightforward and successful.[156] Reasons for failing recombinant membrane protein synthesis can be found in general aspects, such as codon usage, protein and mRNA stability, and differences in glycosylation patterns (reviewed in Section 4.2). Next to that, membrane protein synthesis can also be influenced by membrane properties of host cells including folding and membrane incorporation machineries, membrane composition, and limited space available for protein incorporation.[156,157] Overproduction of membrane proteins was shown to be toxic for host cells.[158] The synthesis of high levels of transmembrane proteins in the cytoplasmic membrane of recombinant *E. coli* has shown to result in the accumulation of cytoplasmic aggregates of the heterologous membrane protein as well as of precursors of native periplasmic and membrane proteins of *E. coli*.[159] Also the incorporation of respiratory chain complexes into the cytoplasmic membrane seemed to be affected. Most probably, all these effects were consequences of the saturation of the protein translocation machinery.[159] Thus, for whole-cell biocatalysis, the protein amount incorporated into membranes is not necessarily the limiting factor, since lower levels, not influencing host cell physiology and metabolism, might be sufficient or even advantageous regarding whole-cell biocatalyst performance. Negative influences on the host might be circumvented by fine-tuning the amount of membrane protein present. Several examples have been described for recombinant membrane proteins applied in whole-cell biocatalysts. For oxyfunctionalizations, the alkane monooxygenase AlkBGT from *P. putida* Gpo1 is a well investigated example.[160] The oxygenase component AlkB, an integral membrane enzyme, derives electrons from NADH *via* soluble redox partners, the rubredoxin AlkG and the rubredoxin reductase AlkT. Its membrane integration enables AlkB to get efficient access to hydrophobic substrates which dissolve well in the hydrophobic core of membranes, while electrons from NADH are delivered from the cytoplasmic side by the redox partners. AlkBGT, with its wide substrate range,[161] was shown to be functional in recombinant *E. coli* for productive whole-cell based hydroxylation of, *e.g.*, natural alkane substrates and fatty acid methyl esters.[162–165] The integral membrane protein XylM from *P. putida* mt-2, another example for the successful application of an integral membrane protein, shows 25% amino acid identity with AlkB and is part of the xylene monooxygenase system XylMA,[166] in which the soluble redox partner XylA transfers the necessary electrons from NADH to the monooxygenase subunit XylM. XylMA was applied in recombinant *E. coli* for the regio-specific benzylic oxygenation of pseudocumene to 3,4-dimethylbezaldehyde (see Table 2, entry 12).[40,41] CYPs originating from eukaryotic organisms typically are membrane-bound proteins as well, being associated to the membranes of the endoplasmic reticulum and depending on membrane-bound or soluble redox partners.[167] Thereby, membrane association is established *via* an N-terminal membrane-anchor. Examples of the use of mammalian as well as microbial cytochrome P450 monooxygenases in recombinant cells are numerous,[8,167,168] but productivities are often limited by low stabilities and coupling efficiencies. CYP71AV1 involved in artemisinin biosynthesis in the plant *Artemisia annua* is an example of a successful application of a membrane-bound plant CYP coupled to cytoplasmic enzymes in an engineered pathway (see Table 2, entry 19).[48] For the production of hydrocortisone, membrane-bound CYPs were introduced into *S. cerevisiae* together with the necessary redox partners (see Table 2, entry 18).[47]

Membrane integrity-related toxicity. Target substrates and products are often toxic to microbial cells. One of the major toxic effects during whole-cell biocatalysis is the loss of membrane integrity.[90,169] Small hydrophobic molecules are able to pass the outer membrane and accumulate in the cytoplasmic membrane. The same is true for the products derived from these compounds. Both, substrates and products, thereby affect membrane fluidity and thus the protective function of membranes as well as vital cellular processes in the membrane, such as the functioning of the respiratory chain and other membrane proteins, *e.g.*, transporters. Finally, toxicity may lead to membrane disintegration and the loss of cofactors or even proteins. Such toxicity effects are detrimental for whole-cell biocatalysis depending on an operational cell physiology. Substrate and product partitioning between the membrane and the aqueous medium, *i.e.*, the partition coefficient $P_{m/aq}$, correlates with the partition coefficient of these compounds in an octanol–water mixture $P_{o/w}$.[90,169,170] In general, hydrophobic compounds with a $\log P_{o/w}$ between 1 and 4 are considered to be toxic to microbial cells. More hydrophobic, larger compounds ($\log P_{o/w} > 4$) are considered non-toxic and do not accumulate in cellular membranes, as they are virtually unable to pass the outer membrane due to their size and hydrophobicity. It has to be mentioned that products in C–O functional group chemistry, especially of oxyfunctionalization reactions, often have a lower hydrophobicity and a higher solubility in water than the substrates they are derived from. Thus, depending on the substrate–product pair, toxic effects may increase during bioconversion. In general, bacteria try to compensate for increased fluidity imposed by hydrophobic compounds by changing the membrane density and composition.[171] Remarkably, some microorganisms, such as certain *Pseudomonas* strains, are tolerant to compounds with a $\log P_{o/w} < 4$ and can even utilize such compounds as growth substrates. These solvent-tolerant organisms are able to adapt to the presence of toxic compounds. The developed solvent tolerance is based on a variety of different mechanisms,[171–173] of which the most important ones relate to the composition of fatty acids (length and *cis*/*trans* isomerization state)[89,174] and phospholipids[175–177] in the membrane, energy-dependent solvent efflux pumps,[173,178] and the formation of vesicles accumulating and exporting toxic compounds (Fig. 23).[179] The use of solvent-tolerant strains in whole-cell biocatalytic processes is discussed in more detail in Section 4.4.

Barrier function of membranes. The natural barrier function of membranes is another key aspect in whole-cell biocatalysis.[180–182] First of all, substrates have to pass membranes in order to be available for enzymatic conversions, but products have to be able to leave the catalyst as well. Thus, the efficiency of whole-cell bioconversions depends on mass transfer over the membrane in both directions. Engineering approaches for the improvement of such mass transfer typically aim at the increase of specific activities of whole-cell catalysts, thus moving processes upwards in the process window shown in Fig. 1. This mass transfer is a function of the cell surface area (A) (or membrane surface area), the concentration gradient over the membrane (C_0–C_1), and a mass transfer coefficient (k).[183] The latter depends on compound-specific chemical properties, such as size and hydrophobicity, but in the case of whole cells also on membrane properties and the presence of (active) up-take and/or excretion mechanisms. It is worthwhile to mention that, compared to cell-free enzymatic reactions, whole cells are less sensitive towards inactivation at phase boundaries (liquid–gas or liquid–liquid) and thus towards strong mixing, which ensures a constant maximal concentration gradient over the membrane.

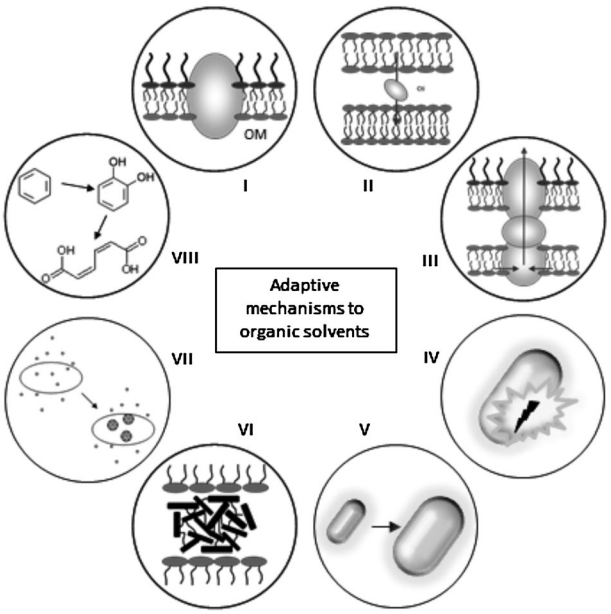

Fig. 23 Adaptive mechanisms protecting cells against toxic effects of organic solvents. (I) Changes in membrane protein pattern; (II) alteration in phospholipid composition; (III) active export of organic solvents by efflux systems; (IV) changes in energy metabolism; (V) changes in morphology; (VI) changes in the cell wall; (VII) vesicle formation; (VIII) transformation or degradation of the solvent. (Adapted from Heipieper *et al.*[92])

Microbial uptake of hydrophilic compounds, which cannot pass the phospholipid bilayers, typically is accomplished by water-filled hydrophilic channels, so called porins, for the crossing of outer membranes and by transporter proteins for the crossing of the cytoplasmic membrane. Substrate transfer *via* porins functions by passive diffusion, whereas different mechanisms are known for transporters including passive diffusion as well as active energy-dependent transport. Such uptake proteins are necessary to supply the cell with nutrients and amino acids, but can also be used for biocatalytic substrates.[182] For the production of 4-hydroxyproline with *E. coli* cells (see Table 2, entry 14),[42] proline is transported into *E. coli* *via* the endogenous transporter PutP functioning as a sodium/proline symporter in the cytoplasmic membrane.[184] The glucose facilitator protein GLF from *Zymomonas mobilis* is an example of a hydrophilic molecule transporter applied in whole-cell biocatalysis *via* heterologous expression.[185,186] Typically, glucose or fructose as carbon and energy sources are transported over the cytoplasmic membrane by the phosphoenolpyruvate

phosphotransferase system, which involves uptake-coupled substrate phosphorylation with the products formed, *e.g.*, glucose-6-phosphate, being the substrates of intracellular central carbon metabolism. The incorporation of GLF enables a parallel transport of glucose without phosphorylation. This glucose can be used as a cosubstrate for recombinant NADPH regeneration systems.[187,188]

Small hydrophobic molecules efficiently enter cells by diffusion through porins and the cytoplasmic membrane, with the concentration gradient as driving force. However, with increasing size and hydrophobicity of the substrate molecule, the lipopolysaccharides on the cell surface and the hydrophilicity of the porins lead to uptake limitations with the outer membrane/cell wall as the main barrier.[182] For these molecules, uptake is limiting biocatalysis and should be optimized in order to ensure maximal substrate availability for the enzymes involved in biotransformation. In general, the availability of low-water-soluble substrates can be maximized by the use of a two-liquid phase setup applying a non-toxic organic water-immiscible phase to supply the substrate and to efficiently extract products.[115,162,189–191] However, for whole-cell biocatalysis, substrate uptake may remain a problem. In order to improve substrate uptake into cells, several approaches have been published. In the case of simvastatine synthesis *via* monacolin J acylation, the membrane-permeability with respect to the substrate was a main criterion determining the synthetic approach chosen.[192] In order to destroy membranes, freeze-thawing, sonication, and high-pressure extrusion have been used.[193,194] Whereas these techniques can be effective in relieving a substrate uptake limitation, they are destructive to the cell. In fact, intact cells are only used to provide the biocatalytically active enzyme in such cases. Other attempts aimed at membrane permeabilization, which can be achieved by chemical treatment of the cells, *i.e.*, applying detergents, solvents, or membrane-destabilizing ion complexing agents. Treatment of *E. coli* cells with different organic solvents or synthetic detergents did improve L-carnitine formation rates with crotonobetaine as the substrate.[195] The addition of Triton X-100 to *Pseudomonas pseudoalcaligenes* improved D-malate production from maleate, but cell lysis was observed as well.[196] Approaches aiming at membrane destruction or permeabilization may be suitable for metabolism independent biocatalysis, but not for enzymatic conversions depending on cell metabolism for cofactor regeneration, enzyme regeneration, and/or (co-)substrate and energy supply. Furthermore, such treatment can lead to cofactor leakage and enzyme destabilization. Engineering of host cell physiology has also proven to be an effective strategy to improve substrate uptake. For this purpose, *E. coli* deletion-mutants with reduced lipopolysaccharide synthesis or lacking Braun's lipoprotein in the outer membrane have been constructed and tested.[155] *E. coli* cells containing recombinant toluene dioxygenase and lacking Braun's lipoprotein showed up to 6-fold increased reaction rates for the oxygenation of the hydrophobic substrates toluene, ethylbenzene, and 2-indanone.[197] For the substrate-limited whole-cell oxyfunctionalization of dodecanoic acid methyl ester and alkanes by means of the alkane monooxygenase AlkBGT, the incorporation of the outer membrane protein AlkL into recombinant *E. coli* proved to be successful, boosting specific oxygenation activities.[165] AlkL is an outer membrane protein from *P. putida* GPo1, which was hypothesized to function as an alkane uptake facilitator in *Pseudomonas* cells growing on these substrates.[198] The presence of AlkL enabled a 62-, 4-, and 28-fold increase in the AlkBGT-based specific oxygenation activity of *E. coli* cells towards dodecanoic acid methyl ester as well as the natural substrates octane and nonane, respectively.[165] AlkL was proposed to function as a hydrophobic porin enabling passive transport over the outer membrane, as was also hypothesized for similar outer membrane proteins.[199–201] Recently, the beneficial effect of AlkL in recombinant *E. coli* cells also was demonstrated for the cytochrome CYP153A6 catalyzed hydroxylation of the monoterpene (*S*)-limonene to (*S*)-perillyl alcohol.[202]

Sub-compartmentalization. Eukaryotes have a more complex cellular structure involving a variety of different membrane structures, *i.e.*, they are compartmentalized by membranes.[203] The resulting compartments include organelles, such as mitochondria, chloroplasts, and peroxisomes, providing internal conditions, which are optimal for specific cellular processes and biochemical reactions. Essential cellular processes such as cofactor regeneration *via* the citric acid cycle and oxidative phosphorylation to gain ATP proceed in mitochondria; photosynthesis in plant cells proceeds in chloroplasts. Peroxisomes are organelles which contain enzymes, *e.g.* oxidases, which produce ROS such as hydrogen peroxide as co-products[135] which are efficiently deactivated in peroxisomes with catalases converting hydrogen peroxide to water and molecular oxygen being the most prominent enzymes involved. The localization of these reactions in separate organelles prevents ROS-related damage to cellular components outside of peroxisomes. The defense mechanisms against hydrogen peroxide and oxygen radicals are of utmost importance for biocatalysis-enabled C–O functional group chemistry and will be further discussed in Section 4.4. For whole-cell biocatalytic processes with eukaryotic cells, sub-compartmentalization may affect biotransformation efficiency, especially when substrates, intermediates, and products have to pass intracellular membrane structures, *e.g.*, when cofactor regeneration and targeted bioconversions proceed in separate compartments. For the production of dicarboxylic acids in *C. tropicalis* (Table 2; entry 11), genes encoding peroxisomal acyl coenzyme A oxidases were deleted in order to circumvent metabolism of fatty acids *via* the peroxisomal β-oxidation cycle and thus redirect the substrate flux towards cytosolic ER-associated ω-oxidation.[127]

Prokaryotes do not feature membrane surrounded organelles. Several other strategies have been investigated for co-localization of different enzymes in order to optimize biocatalysis. *E.g.*, a DNA scaffold binding sequentially operating enzymes increased the efficiency of recombinant *E. coli* as a multistep biocatalyst for the synthesis of resveratrol, 1,2-propanediol, and mevalonate.[204] Enzyme display on the surface of microbial cells was also established, *e.g.*, for CYPs.[205–207] However, several advantageous

aspects of the use of whole cells, such as cofactor and energy supply, efficient synthesis, (re)activation, and folding of enzymes and prosthetic groups, and coupling of several enzymatic steps remain challenging for surface display of enzymes in large-scale production systems. Prokaryotes do synthesize proteinaceous microcompartments encapsulating functionally related enzymes,[208,209] with carboxysomes in cyanobacteria as a prominent example.[210,211] Carboxysomes contain two sequentially operating enzymes, carbonic anhydrase and ribulose biphosphate carboxylase oxygenase (RuBisCO),[212,213] which play a key role in the carbon fixation *via* the Calvin cycle. More recently, so-called polyhedral microcompartments have been described in *Salmonella* and *Citrobacter* species, which contain enzymes for the utilization of 1,2-propanediol[214,215] or ethanolamine.[208] These 'bacterial organelles' are proposed to be useful for biocatalytic purposes when applied as molecular concentrators enhancing biocatalysis.[216] Recently, the formation of an ethanolamine utilization microcompartment from *Salmonella enterica* was successfully established in recombinant *E. coli* including the functional targeting of heterologous proteins to the recombinant microcompartment.[209]

In conclusion, the different cellular compartments offer a wide range of functionalities, making the microbial cell a highly attractive type of biocatalyst, which can be considered a natural and efficient type of enzyme membrane reactor, but also pose challenges mostly related to substrate mass transfer, for which, however, promising solutions have been found.

4.2 Catalyst synthesis and maintenance

A major advantage of microbial over chemical catalysts is their ability to renew themselves. No tedious production processes are necessary to obtain highly regio- and enantioselective catalysts. Considering the complete cell as the catalyst, synthesis and regeneration is simply achieved by cell division at the expense of cheap and readily available resources, mainly consisting of a carbon source (*e.g.*, carbohydrates, glycerol, or organic acids), a nitrogen source (*e.g.*, ammonia, ammonium salts, or amino acids), phosphorous (phosphates), sulfur (sulfates), and metal ions (Na, K, Mg, Ca, Fe, Mn, and Cu). High-cell densities up to 150 g_{CDW} L^{-1} can be achieved with various types of microorganisms applying the right medium and feeding strategy.[217] Such cultivation techniques are of particular interest for (recombinant) protein production.[218–221] For efficient C–O functional group chemistry, however, cell division and the accompanying increase in biomass is only desired to a certain degree, since cell growth and maintenance may energetically compete with the biocatalytic reaction and the biomass itself is a by-product reducing the yield of the target product on the supplied nutrients. For the metabolism-independent reduction of ketones to optically pure alcohols using the enzyme-coupled cofactor regeneration approach in recombinant *E. coli* (see Table 2, entry 4), cells were harvested after cultivation and stored at −20 °C.[25] After thawing, cell growth was prevented by resuspension in a reaction medium consisting of glucose containing phosphate buffer lacking nitrogen. Thereby, ketone reduction to enantiopure alcohol typically required 1–1.5 equivalents of glucose for sufficient nicotinamide cofactor regeneration.[25] The production of L-malic acid (see Table 2, entry 3) is also uncoupled from cell growth.[33] After resuspension/immobilization, the cells were permeabilized and thus used in a non-living state.[24,74] In the case of metabolism-independent reactions, the efficiency of whole-cell biocatalysts easily and efficiently produced *via* high-cell density cultivation depends on the activity, stability, and concentration of the biocatalytically active enzyme(s) ''immobilized'' in the confined interior of the cell.

Biomass formation and production phases can also be separated for biotransformations depending on the cell metabolism. This can either be achieved by simply changing the extracellular biotransformation substrate concentration as in the case of 6-hydroxynicotinic acid production (see Table 2, entry 6)[28,29] or by the introduction of a growth limitation (*e.g.*, lack of nitrogen or phosphorous), thereby transferring the growing cells into a resting, but metabolically active state, enabling high specific activities and yields on energy source.[115,222–225] The performance of this resting cell approach again depends on the intracellular activity, stability, and concentration of the enzyme of interest, and also on the efficiency and stability of the metabolism. Considering the maintenance requirements of resting *E. coli* cells, maximum NAD(P)H yields of 10.3 mol $mol_{glucose}^{-1}$ were calculated for a glucose uptake rate between 1.4 and 2.4 mmol g_{CDW}^{-1} h^{-1}, thus providing metabolic NAD(P)H regeneration rates sufficient for specific activities of 367 U g_{CDW}^{-1} for biocatalytic redox-reactions assuming an equimolar NAD(P)H/product stoichiometry (see Section 4.3 for further metabolic considerations).[226] The most efficient biocatalytic whole-cell reactions for C–O functional group chemistry with recombinant resting cells based on oxygenases are reported for the hydroxylation of propane (118 U g_{CDW}^{-1}),[224] the terminal hydroxylation of methyl nonanoate (174 U g_{CDW}^{-1}),[163] and the hydroxylation of pseudocumene (252 U g_{CDW}^{-1}; Table 2, entry 12).[136] Also for dehydrogenase-based biocatalysts, activities of more than 250 U g_{CDW}^{-1} were reached.[227] However, for redox-biocatalysis, resting cells often lose their activity faster than growing cells.[41,115,228–232] Reasons for a faster loss of activity can be an intracellular NAD(P)H shortage caused by a reduced regeneration rate due to an adaptation of the cellular metabolism to the non-growth status and/or decreasing active enzyme amounts.[17,232] Furthermore, (by-)product-related inhibitions of the metabolism and/or enzymes of interest may be more pronounced in resting cells due to their restricted self-regeneration and stress-handling capacity.[115] Whereas metabolism-related issues can be addressed by metabolic engineering or the introduction of an additional cofactor regeneration system, a low stability of the enzyme of interest favors the application of growing cells. Although the product yield on carbon and energy source will be reduced, since respective substrates will partially be transformed to biomass instead of complete catabolic oxidation to CO_2 with the respective high yields of energy/reduction equivalents, constant regeneration of enzymes responsible for the desired reactions

may result in a more stable biocatalyst and higher product titers, moving respective processes to the right in the process window shown in Fig. 1. Additionally, mechanisms coping with biocatalysis-related stress (*e.g.*, substrate/product toxicity/inhibition, formation of reactive oxygen species – oxidative stress) operate more efficiently in growing cells (reviewed in Section 4.4).[17,115]

Although the microbial cell as a whole can be considered as a catalytic unit, enzymes catalyzing the target reactions deserve special attention. In general, the microbial cell takes care of gene expression, *i.e.*, enzyme synthesis *via* transcription and translation. In wild-type strains, gene expression is tightly regulated *via* different mechanisms in response to changing environmental conditions, cell–cell interactions, or intracellular alterations.[233–240] Recombinant plasmid-based or genome-integrated gene expression ideally can be controlled and induced independently of the host regulatory network, resulting in high and/or fine-tuned expression levels (overexpression) and thus high recombinant enzyme amounts. A vast array of different promoter systems with variable strength is available, constituting a so-called synthetic biology toolbox.[241–248] Depending on the purpose and the reaction system, inducible or non-inducible (constitutive) promoters can be utilized, the latter if (over-)expression of the respective genes has no detrimental effect on the whole-cell biocatalyst. However, overexpression can be challenging, as it is not necessarily directly proportional to copy number and/or promoter strength and may fail at the transcriptional or translational level or lead to the formation of inactive enzymes (inclusion bodies). This is especially critical, if the gene originates from a different phylum or even from a different domain of life.[249] Codon optimization (adaptation of the gene sequence to the codon usage of the heterologous host) is a valuable tool for improving expression levels.[250–254] Today, this technique is frequently applied, since longer DNA-sequences can also be synthesized at reasonable and still decreasing prices. All along the process from initiation of transcription to the correctly folded, active protein, protein synthesis may be enhanced in respect of transcriptional regulation, RNA stability, initiation and elongation during translation, and folding and processing of the amino acid chain. Furthermore, the final protein can be subject to substantial degradation by proteases[249] and, in case plasmid-based expression systems are used, structural and segregational plasmid stability have to be considered.[255]

Targeted modifications on the gene level can not only be applied to optimize expression levels. Moreover, this technique can be used for protein engineering in order to obtain an enzyme with desired properties (*e.g.*, high activity and stability, regio- and/or stereoselectivity, substrate scope, improved coupling).[256–263] This may be achieved by random mutagenesis and subsequent screening or selection, semi-rational approaches, or rational gene design. Novel protein functions can be created by *de novo* design from scratch, resulting in enzymes catalyzing reactions for which no naturally occurring counterparts exist.[264–268] Changes addressing the DNA level can be assigned to the field of synthetic biology. The tremendous progress in synthetic biology and computer-aided design-tools, together with the increasing number of available genome-scale metabolic models and the strong drive of researchers all around the globe to understand metabolic and regulatory networks on a systems level, nowadays allow going a step further than just introducing a (designed) gene in a heterologous host and assure its expression. Metabolic engineering developed from a sequential approach with consecutive single target identifications and optimizations to a more holistic and systematic field (systems bio(techno)logy).[5,147,254,269–274] In order to maximize the flux from substrate to product, Yadav *et al.* proposed four targets to be addressed during a holistic metabolic engineering strategy: (i) enhanced substrate uptake (see Section 4.1), (ii) reduction of fluxes to side-products, thereby improving precursor supply (see Section 4.3), (iii) balanced expression of the heterologous pathway genes, and (iv) shifting reaction equilibria by optimized product secretion.[275] As demonstrated for the biosynthesis of hydrocortisone and artemisinic acid (see Table 2, entries 18 and 19),[47,148] the development of ''designer bugs'' by metabolic engineering enables the production of high value added products from cheap resources making use of genetically engineered microbial whole cells. This is especially true for secondary metabolites. Besides the rational biocatalyst design, it is also crucial to optimize the bioprocess for economically feasible production processes (see Section 5).[5] Thereby, biocatalyst and process engineering are ideally done in parallel with a high level of mutual interaction.

As outlined above, synthesis and maintenance of the catalyst is crucial for both metabolism-linked and metabolism-independent whole-cell bioprocesses. Therefore, functionalities related to catalyst synthesis and maintenance do not only include the natural reproduction mechanisms of microbial cells and their enzymatic machinery, but also a vast array of genetic engineering strategies to enable and improve the production of a target compound by means of whole cells in an economically and ecologically feasible process.

4.3 Metabolism and its use for energy and precursor supply

The metabolism of any organism is responsible for production, maintenance, and destruction of organism constituents and for energy supply. Typically, the heterotrophic metabolism is divided into the oxidative degradation of organic substrates yielding reducing equivalents and energy (catabolism) and the synthesis of biomass constituents at the expense of energy and reducing agents (anabolism). Furthermore, redox metabolism, which is crucial for biological C–O functional group chemistry, is defined as the sum of biochemical modules, in which redox equivalents are created or consumed.[94] Following these definitions, the major metabolic modules of microorganisms involved in whole-cell biocatalysis are discussed in this section. In particular for metabolism-linked biotransformations and fermentations, it is important to have a close look at the energy and redox metabolism, which has a direct influence on biocatalyst efficiency.

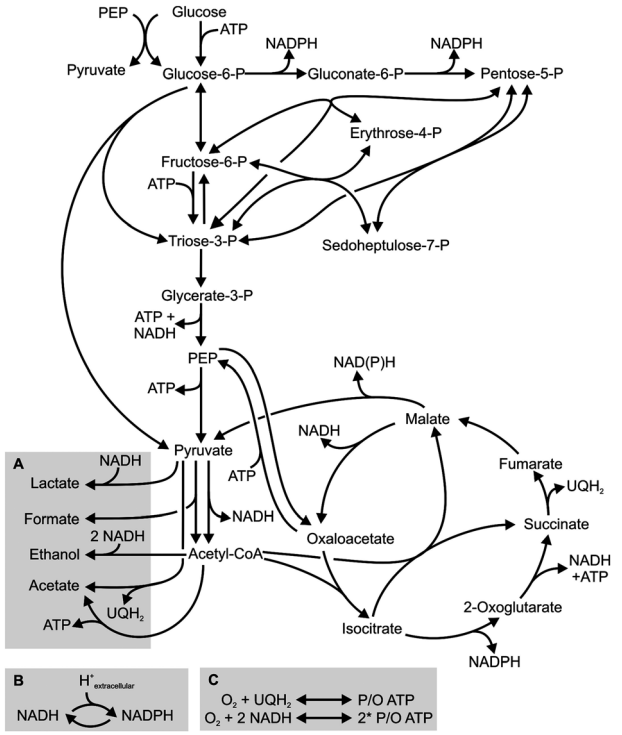

Fig. 24 Central carbon metabolism of *E. coli*. The redox cofactor utilizing reactions are indicated. The fermentative pathways (A), the two transhydrogenase reactions (B), and the respiratory chain (C) are shaded in gray. Only consumed or produced reduction equivalents in the form of NADH, NADPH, or $FADH_2$-derived UQH_2 (reduced ubiquinone) and energy equivalents in the form of ATP or $H^+_{extracellular}$ (for proton gradient exploitation) are indicated. For simplicity, the corresponding reactants (NAD^+, $NADP^+$, UQ, ADP, $H^+_{intracellular}$) are omitted. Abbreviation: P, phosphate. (Taken from Blank *et al.*[94])

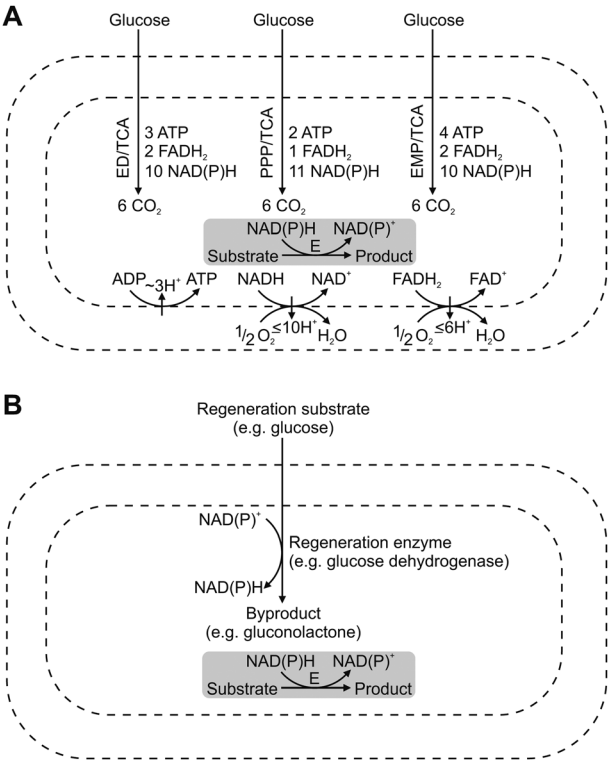

Fig. 25 Energy generation in a living cell (A) and in a dead cell containing a regeneration system (B) coupled to an NAD(P)H-dependent biocatalytic reaction. For the living cell, the three main pathways of oxidative glucose catabolism are shown. ED = Entner–Dourdoroff pathway; PPP = pentose phosphate pathway; EMP = Embden–Meyerhof–Parnas pathway; TCA = tricarboxylic acid cycle. (Adapted from Bühler *et al.*[277])

The central carbon and energy metabolism constitutes the most pivotal metabolic module, being responsible for the generation of energy (*e.g.*, ATP), redox equivalents, and precursors for biomass formation (Fig. 24). In microorganisms, three major pathways are utilized to convert glucose to pyruvate: the glycolysis (Embden–Meyerhof–Parnas pathway, EMP), the pentose phosphate pathway (PPP), and the Entner–Doudoroff pathway (ED). The tricarboxylic acid (TCA) cycle is the major pathway downstream of pyruvate during aerobic growth, whereas the fermentative pathways are active under anaerobic conditions. During aerobic growth, theoretically a maximum of 24 reducing equivalents (electrons) in the form of 12 molecules of NADH, NADPH, or $FADH_2$ is obtained per molecule of glucose (Fig. 25A).[94] With an active EMP pathway, glyceraldehyde-3-phosphate dehydrogenase is the enzyme showing the highest NADH formation rate during aerobic as well as anaerobic growth of *E. coli*. Many organisms lack this route and use the ED pathway instead as the main catabolic pathway with both glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase showing high NADH formation rates.[94,276] Furthermore, aerobic metabolism profits from the high oxidation capacity of the TCA cycle generating NAD(P)H and $FADH_2$ in three and one individual enzyme catalyzed reactions, respectively. Due to the lack of an external electron acceptor (*e.g.*, O_2) during fermentative growth, the formation of reduced redox cofactors needs to be strictly balanced by substrate reduction. Thereby, in fermentative pathways, intermediates of the central carbon metabolism act as electron acceptors (*e.g.*, pyruvate or acetaldehyde), enabling a closed redox balance. During fermentative growth, the energy gain, typically given as amount of ATP formed per substrate consumed, generally is low and based on substrate-level phosphorylation.

Besides energy gain by bacterial carbon catabolism, energy is also wasted in living cells. Such energy spilling occurs mainly by overflow metabolism (*e.g.*, aerobic acetate formation by *E. coli* or ethanol formation by *S. cerevisiae*) reducing the NAD(P)H yield, metabolic shifts, uncoupling of proton gradient and ATP synthesis, and futile cycles as a sacrifice for being prepared for changing conditions. Additionally, maintenance of cellular functions consumes energy.[278] Transhydrogenases constitute an important module in redox metabolism, catalyzing the hydride transfer between the two nicotinamide cofactors NADH and NADPH.[279] Usually, at least one of the two forms of transhydrogenases (*i.e.*, a soluble, non-energy-dependent and a membrane-bound, proton-translocating form) can be found in an organism, with the prominent exception of *S. cerevisiae*, facilitating an on-demand delivery of the required form of redox cofactors. Biomass constituents and secondary metabolites are produced in anabolic pathways from 12 precursor

molecules originating from the central carbon metabolism at the expense of energy (ATP) and redox cofactors.[94] Such a significant drain of redox cofactors and energy in growing cells is expected to influence biocatalysis, if it depends on cellular metabolism and is running at high rates. During respiratory growth, oxidative phosphorylation is the major pathway competing with redox cofactor-dependent biocatalysis. The electron transport chain (ETC) is located in the prokaryotic cytoplasmic membrane or the inner mitochondrial membrane of eukaryotes and constitutes a highly efficient pathway to generate ATP by transferring electrons from a donor (NADH, succinate, or others) to an acceptor (O_2 under aerobic conditions and fumarate, nitrate or others under anaerobic conditions). This electron transfer is coupled to the transfer of protons across the membrane, thereby generating an electrochemical gradient, which is exploited by the ATP synthase to generate ATP. The first enzyme in this pathway is a membrane-bound NADH dehydrogenase possessing a high affinity (low K_m) for NADH,[280–282] which often gives the NADH dehydrogenase a competitive advantage over most biocatalysis-related NADH-dependent oxidoreductases.

The pathways outlined above offer various engineering targets to enable and optimize the fermentative or biotransformative synthesis of a large variety of products ranging from small molecules to macromolecules.

Redox biocatalysis in living cells profits from the cellular capacity to regenerate redox cofactors, typically NAD(P)H, *via* the central carbon metabolism. Depending on the microorganism used, the maximum metabolic NAD(P)H regeneration capacity of growing cells is a critical parameter determining the! economic and environmental feasibility of a biocatalytic process. For different microorganisms, the maximum metabolic NAD(P)H regeneration capacity of cells growing at 30 °C on glucose was estimated to range from 126 to 2218 U g_{CDW}^{-1}, with a value of 575 U g_{CDW}^{-1} for *E. coli*.[276,283] These regeneration rates increase to 300 to 3150 U g_{CDW}^{-1} in resting cells, with a value of 1125 U g_{CDW}^{-1} for *E. coli*, assuming a maximum yield of 10 mol NAD(P)H per mol of glucose catabolized to CO_2 and a glucose catabolism capacity for resting cells corresponding to the glucose uptake rate of growing cells involving the same acetate formation rate. Thus, it is assumed that all glucose flowing into biomass synthesis in growing cells becomes available for cofactor regeneration in resting cells.[283] Duetz *et al.* estimated a value in a similar range (720 U g_{CDW}^{-1}) for growing *E. coli*.[13] At a non-maximal glucose uptake rate of 2.4 mmol g_{CDW}^{-1} h^{-1} giving an optimal yield on glucose (no acetate formation) and considering maintenance energy demands, Blank *et al.* calculated a value of 367 U g_{CDW}^{-1} for resting *E. coli*.[226] Some microorganisms are able to react on an increased demand for energy and cofactors by a significant up-regulation of energy metabolism. The solvent-tolerant *P. putida* strains DOT-T1E and S12 were shown to boost their NAD(P)H regeneration rates up to 8-fold as a response to solvent stress.[93] Such compensation of increased energy requirements to sustain solvent-tolerance mechanisms involved a 1.6–2.5 fold increase of the specific glucose uptake rate and a flux redistribution from anabolism to catabolism with a biomass yield reduction by 35–70%.[89,92] If exploitable, such a high energy and NAD(P)H (re)generation capacity is of high interest for redox whole-cell biocatalysis.

As discussed above, metabolic cofactor regeneration may become limiting, if the target reaction runs at high rates and has to compete with NAD(P)H demands for maintenance and biomass formation (*e.g.*, oxidative phosphorylation).[278] Metabolic engineering strategies aim at the avoidance of the formation of incompletely oxidized metabolic byproducts (metabolic products other than CO_2) and of energy spilling *via* futile cycles as well as at the reduction of cellular maintenance energy demands. Engineering approaches include the increase of reduced cofactor yields on energy source and respective regeneration rates by metabolic flux redistribution, which can be achieved by up- or down-regulation of gene expression levels as well as the deletion of genes involved in carbon and NADH metabolism.[226,284,285] Such approaches are exemplified in Sections 3.2 and 5 on the basis of biocatalytic styrene epoxidation. Due to its essential role in redox metabolism, the ETC also constitutes an important engineering target to improve biocatalysis involving redox cofactors, *e.g.*, by reducing the activity/affinity of the ETC for NADH reduction or by increasing the ETC efficiency in terms of protons translocated per reducing equivalent consumed. The redirection of the electron flow by deletion of an ETC-related gene encoding for cytochrome *bd* oxidase significantly reduced maintenance demands and increased the riboflavin production in *Bacillus subtilis*.[286]

In addition to metabolism-related energy and cofactor consumption, energy dissipation can be increased by several biocatalysis-related phenomena. Cellular stress metabolism typically leads to increased maintenance metabolism. Such stress can be caused by substrate and product toxicity, enzyme toxicity, and solvent toxicity.[277] In case the biocatalytic reaction is energy-dependent, the reaction itself influences the energetic state of the cell. For many oxidoreductases, further energy consumption can occur *via* uncoupling of redox cofactor consumption and product formation or undesired side reactions (*e.g.*, overoxidation or unspecific oxidation).[18,134,226,287,288]

In contrast to the complex situation concerning energy and cofactor supply in living cells, the functionality of dead cells carrying the biocatalytically active enzyme of interest and additionally a heterologous cofactor regeneration system is much simpler (Fig. 25B). However, the yield of reduced nicotinamide cofactors on regenerative cosubstrate, *e.g.*, glucose with glucose dehydrogenase as NAD(P)H regenerating enzyme system, is low in comparison to the complete oxidation of glucose to CO_2 *via* the central carbon metabolism. Especially for low- and medium-priced bulk and fine chemicals, the product yield on carbon and energy source is a critical parameter determining the economic and environmental feasibility of a biocatalytic process.

Besides the importance of the central carbon metabolism for energy, redox cofactor, and cellular precursor supply, biocatalytic reactions can directly depend on central carbon metabolites serving as cosubstrates for the biocatalytic target reaction.

The hydroxylation of L-proline to *trans*-4-hydroxy-L-proline with recombinant *E. coli* containing the proline-4-hydroxylase from *Dactylosporangium* sp. strain RH1 requires stoichiometric amounts of 2-oxoglutarate, an intermediate of the TCA cycle (see Table 2, entry 14).[42] In a similar approach, (2*S*,3*R*,4*S*)-4-hydroxyisoleucine was produced from L-isoleucine by means of an L-isoleucine dioxygenase (IDO)-carrying recombinant *E. coli* strain.[289] IDO also uses 2-oxoglutarate as cosubstrate and electron donor, resulting in the formation of succinate and CO_2.[290] In the TCA cycle, succinate is produced either from 2-oxoglutarate *via* succinyl-CoA or *via* the glyoxylate-shunt pathway, in which isocitrate is converted to glyoxylate and succinate (Fig. 24).[291] An *E. coli* mutant was generated, containing a deletion of both succinate formation routes and the organism was able to restore succinate formation and TCA cycle activity by the introduction of IDO, thereby forcing the metabolism to integrate the biocatalytic reaction in the metabolic reaction network.[289] This approach impressively demonstrates how the central carbon metabolism can be engineered to efficiently support the production of a target compound.

In general, the natural objective of a cell to optimally exploit its energy metabolism for optimized growth and maintenance is often not in line with bioprocess objectives, where the maximization of target product formation is aimed at. However, recent developments and novel approaches in metabolic engineering, systems biotechnology, and synthetic biology progressively allow the efficient implementation of desired reactions in and the adaptation of the complex metabolic network. Thereby, the tremendous flexibility, diversity, and robustness as well as the often high capacity of microbial metabolic pathways can be exploited, moving processes upwards in the process window shown in Fig. 1.

4.4 Prevention of catalyst deactivation/inhibition

With respect to the application of enzymes to perform C–O functional group chemistry, whole-cell biocatalysts often profit from a higher stability as compared to isolated enzymes. The styrene monooxygenase StyAB, which has been applied in isolated form[21,292] as well as in whole cells of *E. coli*[36,37,53,115] (see Table 2, entries 1 and 10) and *Pseudomonas* strains,[54,93,293] can be mentioned as an example. Whole cells have shown to be superior over the cell-free processes, especially with respect to stability, but also productivity and product concentration (see Section 5 and Table 3). For oxygenase-based processes such as the styrene epoxidation, this not only is based on the intracellular environment able to regenerate the enzyme and providing a confined space with high catalyst and metabolite (*e.g.*, NADH) concentrations, but also due to the capability of cells to degrade reactive oxygen species such as peroxides and oxygen radicals. However, also in whole-cell biocatalysis, the stability of the catalyst is an issue, which often limits the reaction efficiency. An activity decrease during biotransformation or fermentation processes can be related to enzyme operation and/or cell physiology. Enzyme operation can be affected by a limited intrinsic stability, catalysis-related deactivation, or enzyme inhibition/deactivation by substrates, products, or other chemicals present. Similarly, cell integrity and metabolism can be affected by toxic effects related to reagents, additives, and/or catalysis (*e.g.* the formation of reactive oxygen species). Thereby, membrane permeabilization and destructive interactions with other macromolecular cell components such as proteins, DNA, and RNA can lead to loss of proteins, cofactors, and/or metabolites and affect cellular metabolic processes resulting in reduced energy supply in the form of ATP, reduced cofactor regeneration, and reduced enzyme synthesis. In order to circumvent such activity loss, nature offers some specific solutions, for which examples will be highlighted in this section. Next to that, solutions on the reaction level have been developed and will be discussed in Section 5.

Some enzymes performing C–O functional group chemistry, *e.g.*, some dehydrogenases, appear to be stable in cell-free or whole-cell setups for a long period of time.[188] Other enzymes, such as oxygenases, are less stable.[17] Enzymes not depending on cellular metabolism (Table 2) can be applied in non-living cells, which may be partially permeabilized while avoiding enzyme and cofactor loss (see Table 2, entry 3). For cofactor regeneration, an enzymatic cofactor regeneration system can be incorporated and operate in such metabolically inactive cells (Fig. 25B; Table 2, entry 4) or cofactors can be supplied *via* the active metabolism of resting or growing cells. For non-living, permeabilized, as well as resting cells, the biocatalytic enzyme cannot be efficiently renewed during the biotransformation, since protein synthesis is absent (dead cells) or impeded (resting cells). For cyclohexanone oxygenation with resting recombinant *E. coli* cells, low enzyme stability of the Baeyer–Villiger monooxygenase has been identified as the main cause for biocatalyst deactivation.[232] In order the prevent deactivation due to enzyme degradation, the use of growing cells is more suitable, since protein synthesis, and thus continuous renewal of catalytic enzymes, will also proceed during the biotransformation (see Section 4.2). For styrene epoxidation by recombinant *E. coli*, the performance of resting and growing cells has been compared in a similar two-liquid phase setup.[37,115] Growing cells appeared to be more stable resulting in higher final product titers. Here, it has to be mentioned that intrinsic stability of the styrene monooxygenase was not the main cause of deactivation, but the sensitivity of cells to product inhibition. Growing cells are typically more flexible in adapting to changing reaction conditions. Some microbial strains can even adapt to and tolerate the presence of toxic solvents, which affect membrane integrity (see Section 4.2).[171–173] These solvent tolerant strains are of high interest for whole-cell biocatalysis.[92] Mechanisms of solvent tolerance are diverse and include solvent extrusion *via* energy-dependent efflux pumps, membrane modifications, and other general stress responses such as protein stabilization and refolding by heat shock proteins (Fig. 23).[294] Solvent tolerant *P. putida* MC2 has been used for the biotransformation of toluene into 3-methyl catechol in a two-phase setup with toxic octanol as the carrier phase (see Table 2, entry 5).[88] This setup allowed the formation of 2.5-fold higher 3-methyl catechol concentrations based on total volume

Table 3 Characteristics of biocatalytic processes for C–O chemistry[a]

Catalyst	Product	Duration [h]	Titer[b]	STY[c] [g L^{-1} h^{-1}]	Catalyst configuration	Process setup	Ref.
Oxyfunctionalization reactions using cell-free systems							
Styrene monooxygenase	Methylstyrene oxide	11	21.2 g L_{org}^{-1}	0.96	Cell-free oxygenase	Batch biotransformation in 2-liquid phase system	21
Choloperoxidase	2-Oxoindole	1	4.96 g L_{aq}^{-1}	4.96	Cell-free peroxidase	Batch biotransformation in buffer/*t*-butyl alcohol	57
Selective C–O chemistry based on whole-cell biotransformations							
Recombinant *E. coli*	10-Hydroxystearic acid	4	49 g L_{tot}^{-1}	12.3	Hydratase in resting cells	Anaerobic batch biotransformation	307
Recombinant *E. coli*	1-(4-Phenoxyphenyl)ethanol	25	201.4 g L_{tot}^{-1}	8.06	Dehydrogenases in resting cells, with cofactor regeneration by recombinant enzymes	Batch biotransformation	25
Recombinant *P. putida* MC2	3-Methylcatechol	55	6.2 g L_{org}^{-1}	0.06	Oxygenase & dehydrogenase in growing cells	Batch biotransformation in a 2-liquid phase system	88
Recombinant *P. putida* MC2	3-Methylcatechol	12	2.1 g L_{org}^{-1} and 0.42 g L_{aq}^{-1}	0.08	Oxygenase & dehydrogenase in growing cells	Biotransformation in membrane reactor with a 2-liquid phase system	308
A. xylosoxidans	6-Hydroxynicotinic acid	25	74 g L_{aq}^{-1}	2.96	Dehydrogenase in resting cells	Batch biotransformation	29
L. kefir	Hexanediol	120	9.5 L_{aq}^{-1}	2.67	Dehydrogenase resting cells	Continuous biotransformation	30
B. bassina	(*R*)-2-(4-Hydroxyphenoxy) propionic acid	120	34.8 g L_{aq}^{-1}	0.29	Oxygenase in growing cells	Batch biotransformation	32
Recombinant *E. coli*	(−)-(1*S*,5*R*)-2-Oxabicyclo-[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-Oxabicyclo-[3.3.0]oct-6-en-2-one	20	20.4 g L_{aq}^{-1}	1.02	Oxygenase in resting cells	Batch biotransformation with solid-phase extraction	110
Recombinant *E. coli*	(−)-(1*S*,5*R*)-2-Oxabicyclo[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-oxa-bicyclo[3.3.0]oct-6-en-2-one	7	4.5 g L_{aq}^{-1}	0.64	Oxygenase in resting cells	Fed-batch biotransformation	35
Recombinant *E. coli*	(*S*)-Styrene oxide	8	72.6 g L_{org}^{-1}	4.54	Oxygenase in growing cells	Batch biotransformation in a 2-liquid phase system	37
Recombinant *E. coli*	(*S*)-Styrene oxide	7	49.6 g L_{org}^{-1}	3.54	Oxygenase in resting cells	Batch biotransformation in a 2-liquid phase system	115
Recombinant *E. coli*	(*S*)-Styrene oxide	100	8.2 g L_{org}^{-1}	0.41	Oxygenase in growing cells	Continuous biotransformation in a 2-liquid phase system	288
Pseudomonas sp. strain VLB120ΔC	(*S*)-Styrene oxide	45	10.2 g L_{org}^{-1}	0.97	Oxygenase in growing cells	Continuous biotransformation in a 2-liquid phase system	293
Pseudomonas sp. strain VLB120ΔC	(*S*)-Styrene oxide	20.5	70.6 g L_{org}^{-1}	1.72	Oxygenase in growing cells	Batch biotransformation in a 2-liquid phase system	54
Pseudomonas sp. strain VLB120ΔC	(*S*)-Styrene oxide	120	45.1 g L_{org}^{-1}	0.25	Oxygenase in biofilm	Semi-continuous biotransformation	309
C. tropicalis	Dicarboxylic acids	120	152 g L_{aq}^{-1}	1.3	Oxygenase & oxidase & dehydrogenase in growing cells	Batch biotransformation in a 2-liquid phase system	133
Recombinant *E. coli*	3,4-Dimethylbenzaldehyde	14.2	36.8 g L_{org}^{-1}	1.3	Oxygenase in growing cells	Batch biotransformation in a 2-liquid phase system	137
Agrobacterium sp. HK13	L-Carnitine	11.1	60 g L_{aq}^{-1}	5.4	Desaturase & hydratase in growing cells	Batch biotransformation	29
Recombinant *E. coli*	4-Hydroxyproline	100	41 g L_{aq}^{-1}	0.41	Oxygenase in growing cells	Batch biotransformation	42
Recombinant *E. coli*	(2*S*,3*R*,4*S*)-4-Hydroxyisoleucine	22	24.1 g L_{aq}^{-1}	1.1	Oxygenase in growing cells	Batch biotransformation	289
P. putida UV4 mutant	Toluene-*cis*-glycol	6	57 g L_{aq}^{-1}	6.65	Oxygenase in resting cells	Batch biotransformation in a 2-liquid phase system	310
Nocardia sp.	Hydroxymethyl simvastatin	110	0.8 g L_{aq}^{-1}	7.3×10^{-3}	Oxygenase in growing cells	Continuous fed-batch biotransformation	311

Table 3 (*continued*)

Catalyst	Product	Duration [h]	Titer[b]	STY[c] [g L^{-1} h^{-1}]	Catalyst configuration	Process setup	Ref.
P. putida GPo1	1,2-Epoxy-7,8-octene	1272	65 g L_{org}^{-1}	1.8×10^{-2}	Oxygenase in growing cells	Biotransformation in membrane reactor with a 2-liquid phase system	312
R. rhodochrous	(*R*)-1,2-Epoxyoctane	120	30 g L_{tot}^{-1},[d]	0.25[d]	Oxygenase in resting cells	Batch biotransformation in a 2-liquid phase system	313
Recombinant *E. coli*	Octanoic acid	5	6.6 g L_{aq}^{-1}	1.2	Oxygenase in growing cells	Batch biotransformation in a 2-liquid phase system	314
Recombinant *E. coli*	Octanoic acid	192	1.9 g L_{aq}^{-1}	0.6	Oxygenase in growing cells	Continuous biotransformation	315
Recombinant *E. coli*	3-Phenyl catechol	9	5.6 g L_{aq}^{-1}	0.62	Oxygenase in growing cells	Fed-batch biotransformation with solid-phase extraction	316
Z. mobilis	Benzyl alcohol	45	0.44 g L_{aq}^{-1}	0.01	Dehydrogenase in biofilm	Semi-continuous biotransformation	317
Fermentative processes involving C–O functional group chemistry							
Recombinant *E. coli*	4-Hydroxyproline	96	25 g L_{aq}^{-1}	0.26	Growing cells	Batch fermentation	43
Recombinant *E. coli*	Propane-1,3-diol	38.6	135 g L_{aq}^{-1}	3.5	Growing cells	Batch fermentation	44
Recombinant *E. coli*	Indigo	72	18 g L_{aq}^{-1}	0.25	Growing cells	Batch fermentation	46
Recombinant *S. cerevisiae*	Hydrocortisone	172	0.02 g L_{aq}^{-1}	1.2×10^{-4}	Growing cells	Batch fermentation	47
Recombinant *S. cerevisiae* Y51	Artemisinic acid	139	2.5 g L_{aq}^{-1}	1.8×10^{-2}	Growing cells	Batch fermentation	48
G. oxydans	Dihydroxyacetone	432	82 g L_{aq}^{-1}	—	Biofilm	Semi-continuous fermentation	318
P. acidipropionici	Propionic acid	2976	15 g L_{aq}^{-1}	—	Biofilm	Semi-continuous, fermentation	319

[a] See text for further details concerning the individual examples listed. Subscripts: aq, aqueous phase; org, organic phase; tot, total volume. [b] Final product titer in the respective phase. [c] Space time yield (STY) with respect to the total production period and total volume. [d] Phase ratio was not reported.

compared to the single aqueous phase setup. Compared to recombinant *E. coli*, the solvent tolerant strain *Pseudomonas* VLB120ΔC showed a higher physiological stability during styrene epoxidation, tolerating higher styrene oxide concentrations (see Section 5).[54] However, non-solvent-tolerant *E. coli* showed higher specific activities and better yields on glucose. The biocatalytic performance of such solvent tolerant strains has to compete with the increased energy demand caused by solvent-tolerance mechanisms,[93,295] such as active transport by solvent efflux pumps. For the development of a whole-cell biotransformation process, the advantages of using solvent-tolerant strains have to be weighed with their disadvantages, such as decreased biomass and product yield on glucose.[54] The possibility to increase the solvent-tolerance of a non-tolerant *E. coli* strain was investigated by heterologous expression of genes encoding for different bacterial efflux pumps.[296] Recombinant *E. coli* containing an efflux pump from *Alcanivorax borkumensis* appeared to be less sensitive towards limonene compared to the wildtype strain. Fermentative production of limonene was increased in the *E. coli* strain carrying the efflux pump, indicating an increased efflux and thus decreased toxicity of limonene.[296] In general, solvent tolerance can help moving the upper boundary of the process window shown in Fig. 1.

Defence mechanisms against oxidative stress constitute another means of metabolically active cells to prevent biocatalyst deactivation. Oxidoreductases, *e.g.*, oxygenases, can cause oxidative stress by uncoupling of O_2 reduction from product formation, resulting in partial O_2 reduction and the formation of ROS such as superoxides and peroxides.[134] ROS are harmful to both the oxygenase[287] as well as the host cell.[297] In cell-free systems, catalases typically are added to protect the enzyme against fast deactivation by hydrogen peroxide.[21,50] The native oxidative stress response of living cells including the action of catalases, peroxidases, and superoxide dismutases protects them against reactive oxygen species.[297] This, together with cofactor regeneration and intrinsic enzyme instability issues, is considered to be an important factor qualifying whole cells as the first choice in oxygenase-based biocatalysis.[19,20,68] For the enantioselective oxidation of 2-hydroxy carboxylic acids by glycolate oxidase, a recombinant *Pichia pastoris* strain was developed which contained next to the glycolate oxidase a recombinantly produced catalase. Using permeabilized cells to avoid substrate uptake limitations, the catalase was utilized to decompose the hydrogen peroxide produced by the oxidase in order to prevent side product formation and deactivation of the glycolate oxidase.[298] In this perspective, it would be

interesting to know whether metabolically active cells without an additional catalase could cope with the hydrogen peroxide produced by glycolate oxidase.

A final example of a natural system preventing deactivation of a catalyst, in this case of the synthetically relevant enzyme, is the *in vivo* reactivation of catechol 2,3-dioxygenase XylE. This dioxygenase catalyzes aromatic ring cleavage in catechols and is part of the TOL-plasmid encoded pathway for the degradation of toluene and xylenes in *P. putida* mt-2. XylE is easily inactivated by catalysis-related oxidation of the iron atom in the active site.[299,300] The *xylT* gene on the TOL-plasmid encodes a ferredoxin which is able to reactivate XylE by bringing the iron atom back to the reduced state.[301,302] Such reactivating components thus help to prevent whole-cell biocatalyst deactivation.

5. Reaction engineering – catalyst and process efficiency and productivity

The previous sections demonstrate the complexity of microbial cells, their functionalities to cope with challenges faced during biocatalytic reactions, and their versatility for selective C–O functional group chemistry. In the following, we will focus on the productivity and efficiency of whole-cell biocatalysis with emphasis on reaction engineering approaches including process concepts and catalyst configurations. For an industrial application of biocatalytic processes, costs for the upstream processing of a reaction constitute a significant share of the overall expenses in a production process. Therefore, availability and storage of the catalyst needs to be carefully considered. Of even more economical importance is the downstream processing of the reaction mixture. Both will not be discussed here and readers are referred to other literature.[6,20,37,189,303–305] It is, however, clear that both upstream and especially downstream processing have to be considered for appropriate catalyst design and reaction engineering, thus following an integrated systems biotechnology approach.[5] *E.g.*, *in situ* product removal has the potential to considerably simplify downstream processing and will be discussed below.

The overall efficiency of a given biocatalytic reaction is mainly determined by the final product titer, the catalyst and process stability (durability), the productivity (STY), and the product yield on carbon source, substrate, or catalyst depending on the respective cost shares.[306] Concerning durability and finally productivity, whole-cell biocatalysts are equipped with several features to cope with biocatalysis-related stress potentially leading to deactivation (as reviewed in Section 4.4). Additionally, a variety of technical solutions is available to prevent catalyst deactivation *e.g.*, by high substrate and/or product concentrations,[52] to maximize substrate availability,[165] and to control reaction specificity.[41] Some of these technical solutions will be highlighted in the following. Table 3 gives an overview on the performance of a variety of bioprocesses developed for specific C–O functional group chemistry, of which some will be discussed below with a focus on reaction engineering concepts. In addition, the operational performance of the selected reactions and processes in terms of STY, product titer, and process duration are visualized in Fig. 26 in order to identify typical ranges along the biocatalytic continuum.

For the hydroxylation of toluene to toluene *cis*-glycol by *Pseudomonas putida* UV4, even comparably low toluene

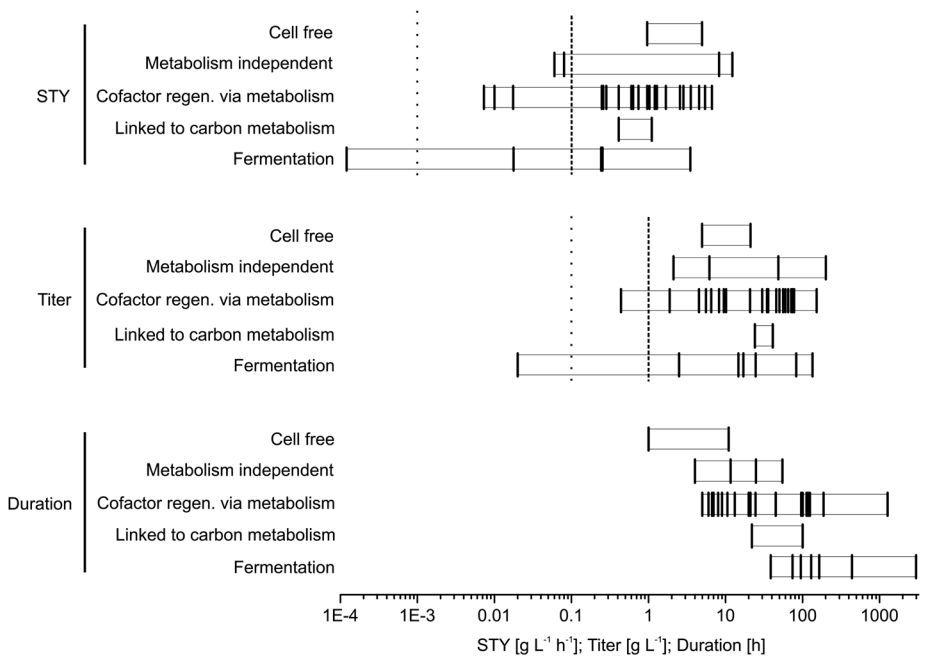

Fig. 26 Operational performance characteristics of the reactions presented in Table 3. Final product titers refer to the product containing phase and space time yields (STYs) are given with respect to total production period and total volume. Performance parameters of the individual reaction examples are indicated as vertical solid bars. Vertical dotted and dashed lines represent the minimum requirements for pharmaceutical and fine chemical production processes, respectively.[7,8,141]

concentrations were found to cause catalyst deactivation.[320] In order to prevent damage to the catalyst, subtoxic toluene concentrations (<0.22 g L^{-1}) were established by feedback regulated substrate addition.[321] Thereby, a toluene feed controlled by the oxygen uptake rate allowed the accumulation of more than 8 g L^{-1} toluene *cis*-glycol in the aqueous medium in 7 h.[322] Similarly, a controlled substrate feed was applied to circumvent substrate toxicity during the Baeyer–Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one using recombinant *E. coli*. In a resting-cell biotransformation at a 200 L scale, 4.5 g L^{-1} product was obtained in 7 h (Table 3).[35] However, for this process, product inhibition finally appeared to be the limiting parameter. The two-liquid phase concept represents another technical solution to prevent catalyst deactivation by substrate toxicity as well as product toxicity. The addition of a non-miscible organic carrier solvent has successfully been used to maximize the availability of hydrophobic substrates and simultaneously extract the product from the aqueous medium *via* ISPR.[37,162,189–191,323] Beside the prevention of catalyst deactivation, this concept facilitates the isolation of products during down-stream processing, thereby enlarging the process window shown in Fig. 1 both by moving the lower boundary (simpler DSP) and the upper boundary (avoidance/reduction of product toxicity). The choice of an appropriate organic solvent depends on several aspects: the solvent should (i) not be toxic to the biocatalyst (featuring a $\log P_{o/w} > 4$), (ii) not be converted or metabolized by the biocatalyst, (iii) provide desirable partitioning characteristics for the substrate and the product, (iv) be suitable for the coupling of an efficient down-stream processing, (v) be available at a low price, and (vi) meet safety regulations (*e.g.* explosion hazard) (see also Section 4.1). If suitable, the substrate itself can function as bulk organic phase as demonstrated for the ω-oxyfunctionalization of fatty acid methyl esters.[165] Furthermore, water immiscible ionic liquids can be applied to function as a substrate reservoir and product sink.[324,325] Solid phase extraction constitutes another powerful ISPR concept to avoid substrate/product inhibition.[326–328] The addition of a substrate loaded adsorption resin (Optipore L-493) for *in situ* substrate feeding and product removal directly to and from the reaction mixture, respectively, enabled a final product concentration of 20.4 g L^{-1} in 20 h for the Baeyer–Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one (Table 3).[110] An external recovery loop including a fluidized bed of the adsorption resin Amberlite XAD-4 was applied for the production of 3-phenylcatechol from 2-phenylphenol, both toxic to the cells, with recombinant *E. coli* JM101 containing the 2-hydroxybiphenyl monooxygenase. Continuous substrate feeding to slightly rate-limiting concentrations and ISPR *via* the resin enabled the production of 5.6 g L_{aq}^{-1} of 3-phenylcatechol in approximately 9 h.[316]

For the production of 3-methylcatechol, the classical two-liquid phase approach and a membrane-mediated two phase setup were evaluated.[88,308] In the latter setup, a hollow-fibre polypropylene membrane module was used to separate the aqueous, catalyst containing phase and the organic phase (consisting of octanol and the substrate toluene). Comparing the overall efficiency of both approaches, similar STY were obtained (0.06 and 0.08 g L^{-1} h^{-1} for the two-liquid phase and the membrane-mediated approach, respectively) (Table 3). The membrane-setup allowed phase ratio engineering in the sense that the carrier solvent volume could be reduced, resulting in an increase of the STY with respect to the organic phase (0.17 as compared to 0.11 g L_{org}^{-1} h^{-1}).[308] In this particular case, accumulation of the toxic product in the aqueous phase to inhibitory concentrations was observed, indicating a mass transfer limitation for the extraction of 3-methylcatechol over the membrane. Increasing the membrane area or the use of a membrane featuring better diffusion characteristics for the product should result in an improved durability and productivity of the process.

The epoxidation of styrene to (*S*)-styrene oxide represents an example for C–O functional group chemistry, which was investigated in detail with respect to reaction engineering applying different catalyst and reaction setups, and will be discussed in the following in order to illustrate the broad scope of reaction engineering targets for the establishment of productive biocatalytic processes. Free[21] and immobilized enzymes[292] as well as suspended cells of recombinant *E. coli* and *Pseudomonas* sp. strain VLB120ΔC growing in fed-batch and continuous mode were applied for this reaction.[37,54,288,293] Furthermore, resting cells[323] and cells immobilized in a biofilm[309] were applied using recombinant *E. coli* and the styrene oxide degradation-negative *Pseudomonas* mutant, respectively. Considering the application of the free enzymes, productivities and the achieved product concentrations (60–80 mM with respect to the total volume) were lower as compared to product concentrations achieved with recombinant whole cells (300 mM) (see Table 2, entries 1 and 10; Table 3).[37,54] Under the process conditions applied, the StyA activity was significantly lower in comparison to short-term assays (8-fold lower), indicating a stability issue *in vitro*. For the application of oxygenases, the stability can typically be increased by their use in whole cells. Due to substrate and product toxicity, the two-liquid phase concept was used for all suspended cell setups as well as for application of free enzyme, whereas membrane-mediated substrate feed and ISPR were applied for the biofilm approach. As outlined above, the application of resting *E. coli* cells resulted in high yields on carbon source and good STY, but in a low durability due to a comparatively fast decrease of catalyst activity due to product inhibition.[323] *E. coli* cells growing in fed-batch mode reached the highest STY which was 1.2-fold higher compared to resting cells and even 2.6-fold higher compared to growing cells of the native host *Pseudomonas* sp. strain VLB120ΔC (Table 3). Due to the higher stability of growing compared to resting cells, the final product concentrations reached were 1.5- and 1.4-fold higher using growing *E. coli* and *Pseudomonas*, respectively (Table 3). Although the solvent-tolerant *Pseudomonas* strain did not reach the high productivity of the *E. coli* strain, the avoidance of side product (2-phenylethanol) accumulation and the tolerance against high substrate and product concentrations was considered beneficial for the process.[54] Solvent tolerance developed by the organism during the biotransformation was

proposed to be responsible for the observed decrease in specific biocatalyst activity. The target reaction(s) may be affected *via* a competition with solvent tolerance mechanisms for redox-cofactors and energy and/or by solvent efflux pump-related active substrate efflux interfering with substrate uptake. Both strains were also used in continuous two-liquid phase processes based on continuous cultivation.[288,293] In general, a continuous setup has possible advantages with respect to the coupling to a continuous downstream processing, reactor volume, and process durability. On the other hand, due to often lower catalyst concentrations and constant medium flow, productivities and product titers, which are relevant for the downstream processing, are typically lower as compared to batch or fed-batch processes. However, when grown in a continuous setup, STYs of 0.41 and 0.97 g L^{-1} h^{-1} over 100 and 45 h and product titers of 4.1 and 5.1 g L^{-1} could be obtained with *E. coli* and *Pseudomonas*, respectively (Table 3).[288,293] The reported process durations can be considered as lower boundaries due to the steady state-related continuous self-regeneration of whole-cell biocatalysts during continuous cultivation.

Such a potentially unlimited stability due to self-regeneration is also given for whole-cell biocatalysts naturally immobilized in biofilms. Biofilms are formed by many bacterial species after attachment to a surface (substratum) and excretion of extracellular polymeric substances (EPS).[329] The cells are embedded in the EPS, resulting in a natural way of catalyst immobilization. At the maturation stage, the biofilm thickness becomes constant, since cell growth and detachment of cells reach an equilibrium. Biofilms are known for increased tolerance against toxic substances and mechanical stress,[330] making them highly attractive for application in chemical syntheses. *Pseudomonas* sp. strain VLB120ΔC was found to efficiently form biofilms and its biocatalytic styrene epoxidation performance was investigated in biofilm setups.[309,331] In a semi-continuous mode, *i.e.*, the organic phase was supplied in batch mode and exchanged every five days, whereas the aqueous phase separated by a membrane serving as substratum was continuously pumped, an average STY with respect to reactor volume (30 mL styrene supplied as organic phase and substrate and 10 mL aqueous volume) of 0.25 g L^{-1} h^{-1} was achieved over 20 days. Such high durability and efficient exploitation of the reaction volume demonstrate the advantages of such (semi-)continuous biofilm-based concepts.

Bearing in mind that the reactions and processes listed in Table 3 represent only a selection and not a comprehensive overview, the operational performances as visualized in Fig. 26 can be used to identify typical ranges for STY, product titer, and reaction duration along the biocatalytic continuum. For the industrial implementation of pharmaceutical and fine chemical production processes, minimal STYs and product titers have been proposed.[7,8,141] These performance limits are indicated in Fig. 26 to illustrate the industrial feasibility of the selected examples. Most of those, irrespective of their allocation along the continuum, fulfil minimal industrial feasibility limits regarding STY and titer. Reaction durabilities tend to increase along the continuum, illustrating the positive effect of catalyst stabilization, which can be achieved by the use of whole and metabolically active cells.

As shown in this section and exemplified for the biocatalytic epoxidation of styrene to (*S*)-styrene oxide, a variety of reaction and process concepts and catalyst configurations can be applied to exploit the potential of whole-cell biocatalysts. Reaction and process development is most efficient when following a systems biotechnology approach, which integrates biocatalyst and reaction engineering and also considers upstream and downstream processing. Finally, scaling aspects such as the limited oxygen mass transfer achievable on industrial scales also need special attention.[13]

6. Conclusions and outlook

Efficient and selective C–O functional group chemistry is challenging for both traditional organic chemistry as well as biocatalysis. The selectivity of enzymes for C–O chemistry is unrivalled and microbial cells are equipped with several functionalities to cope with the challenges arising for these demanding reactions. Cellular membranes, the supply of energy and precursors by cellular metabolism, cell growth and maintenance, and the mechanisms preventing catalyst deactivation have to operate in a concerted manner in order to fully exploit the potential of microbial whole cells for industrial production processes. These functionalities as introduced in this review represent the central components of whole-cell catalysts, enabling the cells to perform a huge variety of synthetically interesting reactions. At the same time, these functionalities are engineering targets to increase the efficiency of a whole-cell biocatalyst and finally a respective bioprocess *via* an integrated approach including the reaction and process engineering levels. The recent developments in and the increasing toolbox of synthetic biology and systems bio(techno)logy now allow the design of tailor-made microbial catalysts for efficient bioconversions. These developments with respect to whole-cell mediated specific C–O functional group chemistry augur well for the future implementation of a variety of novel highly efficient and sustainable bioprocesses in the chemical industry.

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