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Manfred Schrewe

Synthetic pathway and process engineering for terminal oxy- and aminofunctionalization via multistep biocatalysis in living microbial cells

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Summary

Biocatalysis shows high potential for the functionalization of unactivated C-H bonds at ambient conditions with absolute chemo-, regio-, and stereoselectivities being unrivalled by any chemical approach. In order to efficiently exploit nature's toolbox towards an industrial implementation, engineering targets on the reaction as well as the catalyst level need to be identified and tackled in a concerted way.

In this thesis, recombinant microbial cells containing the alkane monooxygenase AlkBGT from *Pseudomonas putida* GPo1 were applied for the ω-functionalization of renewable fatty acid methyl esters (FAMEs). The resulting bifunctional products serve as building blocks for polymer syntheses. AlkBGT-containing *Escherichia coli* were shown to convert FAMEs with 5 to 12 carbon atoms in the alkyl chain giving the highest oxyfunctionalization activities (104 U g_{CDW}^{-1}) for nonanoic acid methyl ester. Kinetic studies revealed that AlkBGT catalyzes a three-step oxidation, yielding ω-alcohols, aldehydes and acids as products. In order to achieve ω-amino-functionalization, AlkBGT-based aldehyde formation was successfully coupled in recombinant *E. coli* with ω-transaminase catalysis (CV2025 from *Chromobacterium violaceum*), enabling the conversion of dodecanoic acid methyl ester (DAME) to 12-aminododecanoic acid methyl ester. Substrate uptake was identified as key factor limiting the conversion of the large and hydrophobic substrate DAME (1.4 U g_{CDW}^{-1}; 0.1 g L^{-1} h^{-1}). Co-expression of the gene encoding the outer membrane protein AlkL relieved the uptake limitation and boosted the activities 62-fold to 87 U g_{CDW}^{-1} allowing productivities of 4-8 g L^{-1} h^{-1} in two-liquid phase biotransformations. Furthermore, the latter reaction engineering concept enabled to control and govern product formation during AlkBGT-based multistep whole-cell biocatalysis. Overoxidation was prevented providing excess of substrate, yielding ω-alcohol as predominant product (up to 52 mM), whereas pronounced substrate limitation using bis(2-ethylhexyl)phthalate as organic carrier solvent resulted in almost exclusive acid accumulation (up to 93 mM). Introduction of the NAD(P)H-independent alcohol dehydrogenase AlkJ, which was shown to catalyze irreversible alcohol oxidation, into *E. coli* containing AlkBGT and AlkL enabled a shift towards the formation of overoxidized compounds in two-liquid phase biotransformations of DAME. This allowed the formation of the aldehyde as predominant product (up to 20 mM).

Via catalyst and reaction engineering, this study sets the stage for the industrial implementation of recombinant microbial cells for terminal FAME functionalizations.

Zusammenfassung

Die Biokatalyse ermöglicht die synthetisch sehr anspruchsvolle, durch traditionelle chemische Synthesen unerreichte, chemo-, regio- und stereoselektive Funktionalisierung von C-H Bindungen unter milden Bedingungen. Um Biokatalysatoren für die großtechnische Produktion effizient nutzen zu können, müssen Reaktionstechnik- und Katalysatoroptimierung kombiniert werden.

In der vorliegenden Arbeit wurden rekombinante *Escherichia coli* zur Herstellung ω-funktionalisierter Produkte aus nachwachsenden Fettsäuremethylestern (FSME) entwickelt und eingesetzt. Die resultierenden bi-funktionalen Produkte stellen wichtige Ausgangssubstanzen in der Polymersynthese dar. FSME mit einer Kettenlänge von 5 bis 12 Kohlenstoffatomen wurden durch die Alkanmonooxygenase AlkBGT in rekombinanten *E. coli* zu ω-Alkoholen und Aldehyden umgesetzt. Die höchsten Aktivitäten (104 U g_{BTM}^{-1}) wurden für Pelargonsäuremethylester erreicht. Kinetische Untersuchnungen zeigten, dass AlkBGT eine Dreischritt-Oxidation über ω-Alkohol und Aldehyd zur Säure katalysiert. Zur Herstellung des ω-Amins aus Laurinsäuremethylester (LSME) wurde die AlkBGT-katalysierte Aldehydbildung erfolgreich intrazellulär an die ω-Transaminase CV2025 gekoppelt. *In vitro* Studien zeigten, dass die Umsetzung des großen und hydrophoben Substrates LSME durch die Aufnahme in die Zelle limitiert wird (1.4 U g_{BTM}^{-1}, 0.1 g L^{-1} h^{-1}). Diese Limitation wurde durch die Koexpression des *alkL*-Gens, welches für ein Protein der äußeren Membran kodiert, stark reduziert. Hierdurch wurde die spezifische LSME-Oxidationsrate in Zwei-Flüssigphasen-Biotransformation um den Faktor 62 auf 87 U g_{BTM}^{-1} erhöht, was Raum-Zeitausbeuten von 4-8 g L^{-1} h^{-1} ermöglichte. In der Mehrschrittoxidation von LSME ermöglichte das Zwei-Flüssigphasen-Konzept zudem die Kontrolle und Steuerung der Produktbildung. Substratüberschuss unterdrückte die Überoxidation, wodurch bis zu 52 mM ω-Alkohol als Hauptprodukt hergestellt wurden. Kontrollierte Substratlimitierung durch den Einsatz von Bis(2-ethylhexyl)phthalat als organische Trägerphase erlaubte die fast ausschließliche Akkumulation der Säure (bis zu 93 mM). Des Weiteren wurde gezeigt, dass die NAD(P/H)-unabhängige Alkoholdehydrogenase AlkJ eine irreversible Alkoholoxidation katalysiert. Mit AlkBGT, AlkL und AlkJ tragenden *E. coli* wurde eine deutliche Verschiebung im Produktbildungsmuster hin zu den Überoxidationsprodukten erreicht, was im Zwei-

Flüssigphasen-System die Produktion des Aldehydes als Hauptprodukt (bis zu 20 mM) ermöglichte.

Durch die kombinierte Entwicklung von Biokatalysator und Reaktionstechnik wurden in der vorliegenden Arbeit die Voraussetzungen für eine großtechnische Anwendung von Ganzzell-Biokatalysatoren für die ω-Funktionalisierung von erneuerbaren FSME geschaffen.

List of abbreviations

2-LP	Two-liquid phase
μ	Growth rate
ω-TA	ω-Transaminase
A	Cell surface area
ADAME	12-Aminododecanoic acid methyl ester
ADA	12-Aminododecanoic acid
ADH	Alcohol dehydrogenase
ALDH	Fatty aldehyde dehydrogenase
AlkB	Alkane monooxygenase
AlkG	Rubredoxin
AlkH	Aldehyde dehydrogenase
AlkJ	Alcohol dehydrogenase
AlkK	Acyl-CoA synthetase
AlkT	Rubredoxin reductase
AOX	Fatty alcohol oxidase
ATP	Adenosine triphosphate
BEHP	Bis(2-ethylhexyl)phthalate
BSA	Bovine serum albumin
BVMO	Baeyer-Villiger monooxygenase
CDW	Cell dry weight
CHMO	Cyclohexanone monooxygenase
CoA	Coenzyme A
CPO	Chloroperoxidase
CSTR	Continuous stirred tank reactor
CYP	Cytochrome P450 monooxygenase
DAME	Dodecanoic acid methyl ester
DDA	Dodecanedioic acid
DDAME	Dodecanedioic acid methyl ester
DCPK	Dicyclopropyl ketone
DMSO	Dimethyl sulfoxide
DOT	Dissolved oxygen tension
DSP	Down-stream processing
ee	Enantiomeric excess
ED	Entner-Doudoroff pathway
EMP	Embden-Meyerhof-Parnas pathway
EPS	Extracellular polymeric substances
ETC	Electron transport chain
FAD/H_2	Oxidized/reduced flavin adenine denucleotide
FAME	Fatty acid methyl ester
FDH	Formate dehydrogenase
GC	Gas chromatography
GDH	Glucose dehydrogenase
GOX	Glucose oxidase
HDA	12-Hydrododecanoic acid
HNAME	9-Hydroxynonanoic acid methyl ester

HPLC	High performance liquid chromatography
HPOPS	(*R*)-2-(4-hydroxyphenoxy) propionic acid
ISPR	*in situ* product removal
k	Mass transfer coefficient
k_{cat}	Catalytic constant (s^{-1})
k_{cat}/K_m	Specificity constant ($M^{-1}\ s^{-1}$)
K_m	Michaelis dissociation constant ($mol_{substrate}\ L^{-1}$)
Km	Kanamycin
K_P	Partition coefficient
KPi	Potassium phosphate buffer
K_s	Substrate uptake constant ($mol_{substrate}\ L^{-1}$)
L_{aq}, L_{org}, L_{tot}	Aqueous, organic, and total volume, respectively
LDH	Lactate dehydrogenase
MCS	Multiple cloning site
MS	Mass spectrometry
NAD(P/H)	(reduced) Nicotinamide adenine dinucleotide (phosphate)
NAME	Nonanoic acid methyl ester
OD_{450}	Optical density at 450 nm
ONAME	9-Oxononanoic acid methyl ester
P4H	Proline-4-hydroxylase
PLP	Pyridoxal 5'-phosphate
PMS/DCPIP	Phenazine methosulphate/2,6-dichlorophenol-indophenol
POPS	(*R*)-2-phenoxypropionic acid
PPP	Pentose phosphate pathway
ProA	γ-glutamylphosphate reductase
ProB	γ-glutamyl kinase
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFPR	*in situ* substrate feeding and product removal
STY	space time yield ($g_{product}\ L^{-1}\ h^{-1}$)
StyAB	Styrene monooxygenase
Tc	Tetracycline
TCA	Tricarboxylic acid cycle
TF	Turnover frequency ($mol_{product}\ mol_{enzyme}^{-1}\ s^{-1}$)
TN	Turnover number ($mol_{product}\ mol_{enzyme}^{-1}\ s^{-1}$)
TodCBA	Toluene dioxygenase
TodD	*cis*-Dihydrodiol dehydrogenase
TodE	3-methylcatechol 2,3-dioxygenase
TTN	Total turnover number ($mol_{product}\ mol_{enzyme}^{-1}$)
ODAME	12-Oxododecanoic acid methyl ester
U	Unit ($\mu mol_{product}\ min^{-1}$)
UQH_2	Reduced ubiquinone
V	Operating volume
V_{max}	Maximum reaction velocity ($U\ g_{CDW}^{-1}$)
V_{max}/K_s	Specificity constant ($U\ g_{CDW}^{-1}\ M^{-1}$)
$Y_{p/x}$	Product yield on biocatalyst ($g_{product}\ g_{CDW}^{-1}$)
$Y_{p/s}$	Product yield on substrate ($mol_{product}\ mol_{substrate}^{-1}$)

1: Introduction

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1.1 Preface

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 favor 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 chapter 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.

1.2 Introduction

The catalytic regio-, stereo-, and chemoselective generation or alteration of C-O functionalities is a basic chemical concept in nature, 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 (Hanefeld *et al.*, 2009), 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 (Liese *et al.*, 2006b). 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. Figure 1.1 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 it was introduced by Woodley and Titchener-Hooker (Woodley and Titchener-Hooker, 1996).

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 chapter covers all issues for 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, Figure 1.2) 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 1.5. 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 all complex, but also simple, enzyme systems used to synthesize materials, chemicals, or even energy carriers of the future.

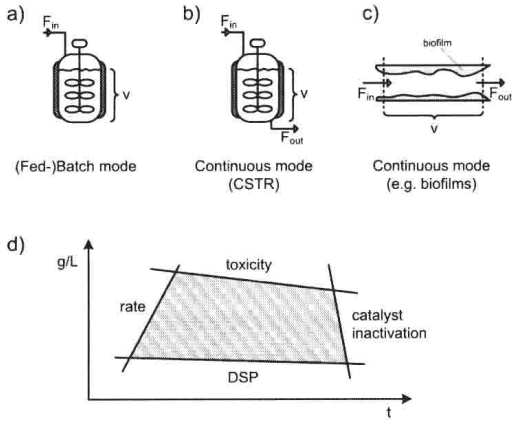

Figure 1.1: Different process concepts applied in biocatalytic whole-cell reactions (a – c) and the process windowwhich defines the boundaries of such processes. v, operating volume; CSTR, continuous stirred tank reactor; DSP, down-stream processing.

1.3 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) (Gardossi *et al.*, 2010). Such quantitative parameters include specific biocatalyst activity and kinetics, yield on biocatalyst (TTN), volumetric productivity, and the product concentration achieved (Kuhn *et al.*, 2010a; Tufvesson *et al.*, 2011). Noteworthy, parameters of whole-cell approaches (may) differ slightly from their equivalents in cell-free processes (Table 1.1). 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) typically is defined as one µmol product formed per minute. In whole-cell biocatalysis, specific product formation rates are commonly calculated related 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 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