Biofilms as living catalysts for fine chemical synthesis: analysis, process design and scale-up

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“Biofilms” presumably the dominant life forms on earth.

பிச்சை புகினும் கற்கை நன்றே.

– ஔவை

To my mom

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I am always indebted to my dad and my family members for their love and encouragement in my every movement.

Thanks Deepa for everything!

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List of abbreviations and nomenclature

Symbol	Abbreviation
3D	Three dimensional
AFM	Atomic force microscopy
ALR	Air lift reactor
ATP	Adenosine triphosphate
BEHP	Bis(2-ethylhexyl) phthalate
BSA	Bovine serum albumin
CFU	Colony forming unit
ConA	Concanavalin A
CLSM	Confocal laser scanning microscopy
CYP	Cytochrome P450
CTC	5-cyano-2, 3-ditolyl tetrazolium chloride
CH_4	Methane
CO	Carbon monoxide
CO_2	Carbon dioxide
Ca^{2+}	Calcium ion
CDC	Centers for disease control and prevention
d	Day
DDAO	7-Hydroxy-9*H*-(1,3-Dichloro-9,9-Dimethylacridin-2-One)
Da	Dalton
DNA	Deoxyribonucleic acid
ee	Enantiomeric excess
ePTFE	Expanded polytetrafluoroethylene
EABR	Electro active biofilm reactor
ESEM	Environmental scanning electron microscopy
EPS	Extracellular polymeric substances
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
FISH	Fluorescent in situ hybridization
FBR	Fluidized bed reactor
g	Grams
g	Gravitational force
g_{cdw}	gram cell dry weight
GC	Gas chromatography
GFP	Green fluorescent protein
H_2	Hydrogen
H_2S	Hydrogen sulfide
HPLC	High performance liquid chromatography
h	Hour
ID	Inner diameter
$k_L{\cdot}a$	Oxygen mass transfer coefficient per unit volume
K_m	Michaelis constant for the substrate affinity of an enzyme
kW	Kilowatt
L	Liter
mL	Milli liter (10^{-3} liter)
µL	Micro liter (10^{-6} liter)
L_{aq}	Volume of aqueous phase in liter
$logP_{O/W}$	Logarithm of octanol-water partition coefficient
min	Minute
mm	Milli meter
mM	Milli molar

Symbol	Abbreviation
MABR	Membrane aerated biofilm reactor
MBR	Membrane biofilm reactor
MFC	Microbial fuel cells
NAD^+/H	Oxidized/reduced nicotinamide adenine dinucleotide
$NADP^+/H$	Oxidized/reduced nicotinamide adenine dinucleotide phosphate
nm	Nanometer
NO_2	Nitrogen dioxide
OD_{450}	Optical density at 450 nm
OD	Outer diameter
O_2	Oxygen
OCT	Optical coherence tomography
PI	Propidium Iodide
PCSBR	Plastic composite supports biofilm reactor
PCS	Plastic composite supports
PBR	Packed bed reactor
PVDF	Polyvinylidene fluoride
PVC	Polyvinyl chloride
PES	Polyether sulfones
Pa	Pascal
P_{min}	Minimum productivity
P_{max}	Maximum productivity
QS	Quorum sensing
rpm	Revolutions per minute
RDBR	Rotating disc biofilm reactor
RM	Raman microscopy
s	Second
SFBR	Segmented flow biofilm reactor
SMABR	Solid support membrane aerated biofilm reactor
SEM	Scanning electron microscopy
sp.	Species
TBR	Trickle bed reactor
T_{min}	Minimum process time
T_{max}	Maximum process time
US*	Uwe Sauer*
U	Activity unit equal to 1 µmol product formed per minute
vol/vol	Volume by volume
V	Volt
VBNC	Viable but not culturable
wt/vol	Weight by volume
W	Watt
µM	Micro molar

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Summary

Biofilms are resilient to a wide variety of environmental stresses. This inherited robustness has been exploited mainly for bioremediation. This thesis addressed the key challenges for the use of biofilms as biocatalysts on biological as well as on technical level. On biological level the question how do catalytic biofilms react to the biologically challenging compound styrene was studied. On technical level, crucial process parameters such as substratum for biofilm growth and oxygen mass transfer have been targeted.

To address the biological question, a reliable toolbox based on a modified flow-cell coupled to confocal microscopy and a GFP-marker system was established. Through a time-resolved, non-invasive and quantitative approach, *Pseudomonas* sp. strain VLB120ΔC biofilm development and its response to the toxic solvent styrene was investigated. Although *Pseudomonas* cells experience severe membrane damage during styrene treatment, they are able to adapt to the toxic conditions and recover. The solvent styrene did not affect the growth rate and overall biofilm structural integrity. Compared to control experiments with planktonic cells, the *Pseudomonas* biofilm adapted much better to toxic concentrations of styrene, as nearly 65% of biofilm cells were not permeabilized (viable), compared to only 7% in analogous planktonic cultures.

For the synthesis of target product (*S*)-styrene oxide on a process level, a unique solid support membrane aerated biofilm reactor was designed, constructed and scaled-up utilizing *Pseudomonas* sp. strain VLB120ΔC growing in a biofilm as biocatalyst. In the optimized system a sintered stainless steel membrane was used as a growth surface and efficient oxygen transfer unit, while a highly stable expanded PTFE (ePTFE) membrane showed the best performance regarding *in situ* substrate delivery and product extraction. With these modifications, the scalability of the system was demonstrated and the solid support membrane aerated biofilm reactor was scaled-up by a factor of 12 with respect to an aqueous phase volume. In the scaled up system, a productivity of 24 g L_{aq}^{-1} day^{-1} could be achieved with an excellent product on biomass yield of 23 $g_{product}$ $g_{biomass}^{-1}$. In total, 46 g of (*S*)-styrene oxide was produced in this system and was subsequently isolated by vacuum distillation (purity: 99%; *ee*: > 99%). The achieved productivity is in a similar range to the small scale biofilm reactor verifying the success of the scale-up. Finally, to test the compatibility of this reactor concept, an equally challenging reaction for limonene

hydroxylation was tested. (*S*)-perillyl alcohol was synthesized at the rate of 7 g L_{aq}^{-1} day^{-1} using *Pseudomonas* sp. strain VLB120 harboring cytochrome P450 monooxygenase. A stable catalytic activity of more than 32 days was achieved before the system was terminated actively.

These results set a new status and open new possibilities for future developments in the field of productive catalytic biofilms.

Zusammenfassung

Biofilme zeichnen sich durch ein hohes Maß an Stresstoleranz aus. Diese Eigenschaft wird vor allem in diversen Verfahren zur Altlastensanierung, bzw. in der Gewässer- und Abluftreinigung genutzt, wo Biofilme eine wichtige Rolle im Abbau von Schadstoffen spielen. In dieser Arbeit wurden Biofilme des Organismus *Pseudomonas* sp. strain VLB120 hauptsächlich für die Synthese von (*S*)-Styroloxid eingesetzt. Dazu wurde zunächst ihre Reaktion auf das toxische Substrat Styrol näher untersucht. Um dieses Verhalten näher untersuchen zu können, musste die dafür notwenige Methodik im Labor etabliert und auf die Gegebenheiten angepasst werden. Hierzu wurde *Pseudomonas* sp. strain VLB120 mit einem fluoreszierenden GFP-Marker versehen und eine Standard-Fließzelle so modifiziert, so dass es möglich war mittels konfokaler Laser-Scanning-Mikroskopie unter „quasi-Prozess-Bedingungen" das Verhalten des Biofilms auf Phänotyp Ebene zu verfolgen. Unterstützt wurde diese Methodik durch eine ganze Reihe an biochemischen Methoden, welche eine genaue Analyse der einzelnen Biofilm-Komponenten ermöglichte. Obgleich die Zellen des Biofilms sehr sensitiv auf die Zugabe von Styrol reagierten und eine schwerwiegende Permeabilisierung der Zellmembran aufwiesen, war es ihnen möglich sich an die Gegebenheiten anzupassen und sich zu erholen. Erstaunlicherweise hatte Styrol keinerlei Einfluss auf die Wachstumsrate und die durchschnittliche Biofilm Struktur. Im Vergleich zu Experimenten mit planktonisch wachsenden Zellen konnte sich der *Pseudomonas* sp. strain VLB120 Biofilm wesentlich besser an die toxischen Styrolkonzentrationen anpassen. Während lediglich 7% der planktonischen Kultur sich vom Styrolschock erholen konnte, lag der Anteil der nicht beeinträchtigten Zellen im Biofilm bei 65%.

Für die Synthese von (*S*)-Styroloxid auf Prozessebene wurde ein spezieller Reaktor entwickelt, welcher über ein kombiniertes Belüftungsmembran/Wachstumsoberflächen Modul verfügte. Eine Stammvariante *Pseudomonas* sp. strain VLB120ΔC wurde als Katalysator eingesetzt. Dieses System wurde genau untersucht und nach einigen Optimierungen auf den Pilot-Maßstab gebracht. Das optimierte System verfügt über ein kombiniertes Belüftungsmembran/Wachstumsoberflächen-Modul aus gesintertem Edelstahl und einer „expanded PTFE" (ePTFE) Membran mit ausgezeichneten Massentransfer-Eigenschaften und Lösungsmittelbeständigkeit zur kontinuierlichen *in situ* Substrat-Zugabe und Produktextraktion. Im Pilot-Maßstab wurde eine Produktivität von 24 g L_{aq}^{-1} T^{-1} erzielt

mit einer hervorragenden Ausbeute von 23 $g_{Produkt}\ g_{Biomasse}^{-1}$. In Summe wurden 46 g reines (*S*)-Styroloxid (Reinheitsgrad 99%; *ee*>99%) produziert, welches per Vakuum-Destillation aus der organischen Phase abgetrennt wurde. Die Maßstabs-Vergrößerung um Faktor 12 ist als erfolgreich zu bezeichnen, da die erreichte Produktivität im ähnlichen Rahmen wie die Leistung des kleineren Labor-Maßstab-Systems liegt.

Um die generelle Anwendbarkeit dieser Technologie auf andere Reaktionen zu demonstrieren, wurde das System zur Herstellung von (*S*)-Perillyl-Alkohol genutzt. Dazu wurde *Pseudomonas* sp. strain VLB120 mit den entsprechenden Genen zur Synthese von Cytochrom-P450-Monooxygenase ausgestattet, welche die Hydroxylierung von Limonen zum entsprechenden Alkohol katalysiert. Auch dieses Produkt konnte mit einer vernünftigen Produktivität von 7 g $L_{aq}^{-1}\ T^{-1}$ über 32 Tage synthetisiert werden, bevor das System aktiv heruntergefahren wurde.

Damit hat diese Arbeit den Grundstein für neue Entwicklungen im Bereich Bioprozessentwicklung mit katalytischen Biofilmen gelegt.