Microbial physiology as an efficiency determining factor in oxidative biocatalysis

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
Doctor of Natural Sciences

presented by
DANIEL PHILIPPE MEYER
Dipl. Biotech.
Ecole Supérieure de Biotechnologie de Strasbourg
born on November 29, 1975
citizen of Aesch, BL (CH)

accepted on the recommendation of
Prof. Dr. Bernard Witholt, examiner
Prof. Dr. Andreas Schmid, co-examiner
Prof. Dr. Sven Panke, co-examiner

Zurich, 2006
Acknowledgements

I wish to express my gratitude to everyone who contributed to making this thesis a reality. I would like to single out my supervisor Andreas Schmid: thank you for the freedom you gave me to work out every idea I’ve had and your guidance to bring them to fruition with a minimum of limits. Moving to Dortmund was a great experience!

I would like to thank Bernard Witholt for giving me the opportunity to start a career in science at ETH and for sharing his enormous experience in biotechnology with me. I am very grateful to Sven Panke and Nikolaus Amrhein for their kindness to accept the responsibility to be co-examiner and chairman for my defense.

I am thankful to Daniel Kuhn and Patrick Kehl who significantly contributed to this thesis by their diploma works: I am happy that I had the opportunity to accompany you on your final step to graduation. I also would like to thank Grit Neumann and Sabine Nussbaum for the pleasant and successful team works, Bruno Bühler and Georgios Ionidis for so many fruitful discussions on the topic of whole-cell biotransformation (“Whole cells rule!!!”), and to Patty Kleinsteuber for the excellent technical assistance and the entertainment during bioreactor experiments. Thanks to Andreas Meyer and Jochen Lutz who were very enjoyable lab mates. Heiko Hayen and Hermann Heipieper are acknowledged for the fruitful collaborations.

I would like to thank Helena Zuber, Christine Wasielewski, and Judith Fadel for having managed all administrative issues, which gave me the time to focus on scientific tasks. I am indebted to Colette Rohrbach and Sammy Majdi for their excellent IT support (my laptop is still running!) and for numerous discussions outside of biotechnology.

I also would like to mention all the other colleagues in Zurich and Dortmund and thank them for providing such a productive working atmosphere and friendly social environment.
I am grateful to my parents Tony and Marianne as well as to my brother Michel and my sister Simone for their support and encouragement throughout my studies. Finally, I would like to thank Sabine for her moral support, her love and patience and for standing by my side in all ups and downs.

This work was financially supported by the European Commission, by the “Deutsche Bundesstiftung Umwelt” (DBU), and by the Center of Applied Chemical Genomics (ZACG).
# Table of contents

## Summary

### Zusammenfassung

Chapter 1: **Introduction**

1.1 General introduction

1.2 Gene expression
   - 1.2.1 Promoters
   - 1.2.2 Limitations of protein overproduction

1.3 Cell metabolism
   - 1.3.1 Avoidance of side-product formation
   - 1.3.2 The use of recombinant host strains

1.4 Cofactor availability

1.5 Oxygen transfer

1.6 Catalyst stability and inactivation
   - 1.6.1 Two-liquid-phase biotransformation
   - 1.6.2 Application of solvent-tolerant bacteria in two-liquid-phase biotransformations
     - 1.6.2.1 Mechanisms of solvent tolerance
     - 1.6.2.2 Suitability of solvent-tolerant strains for industrial applications

1.7 Conclusions and future prospects

1.8 Scope of the thesis

Chapter 2: **Suitability of recombinant Escherichia coli and Pseudomonas putida strains for selective biotransformation of m-nitrotoluene by xylene monooxygenase**

Chapter 3: **The catalytic efficiency of recombinant Pseudomonas putida for the selective oxidation of aromatic and heteroaromatic compounds**
Chapter 4: Growth and physiological response of *Escherichia coli* JM101 in the presence of 1-decanol 83

Chapter 5: Towards the production of (R)-phenylacetylcarbinol by the multistep biotransformation of toluene coupled to the central metabolism of *Escherichia coli* 103

Chapter 6: Concluding remarks and outlook 129

Literature 133

Curriculum vitae 153
Summary

Bacteria are often exposed to unnatural conditions during biotransformations, which can affect cell metabolism and thus catalytic efficiency. Investigating the influence of the physiology of different host strains on the efficiency of oxidative biotransformations was the topic of this thesis.

The xylene monooxygenase (XMO) catalyzed multistep oxidation of \( m \)-nitrotoluene to the corresponding alcohol, aldehyde, and acid was influenced by the enzymatic background activity of *Pseudomonas putida* and *Escherichia coli* host strains. The nitro group of substrate and products was reduced to the amine group, and the carbonyl group of \( m \)-nitrobenzaldehyde was reduced or oxidized to the hydroxyl or carboxyl group, respectively. A slower accumulation of amines, which reversibly inhibited XMO, favored the use of *P. putida* rather than *E. coli* strains as hosts in this application.

We constructed a series of plasmids for XMO and styrene monooxygenase overproduction in different bacterial species. As a result of a more varying gene expression level, monooxygenase activities were less reproducible for biocatalysts based on solvent-tolerant *P. putida* DOT-T1E and *P. putida* S12 than on solvent-sensitive *P. putida* KT2440 and *E. coli* JM101. In contrast to *P. putida* DOT-T1E, *E. coli* JM101 grew with a second phase of 1-decanol without long-term adaptation, which makes this strain an interesting candidate as host in such two-liquid-phase systems. Analysis of the membrane revealed no typical solvent tolerance response of the *E. coli* JM101 bacteria on the level of lipid and fatty acid residue composition as a result to incubation with 1-decanol/buffer. The low reproducibility of catalytic performance and protracting adaptation procedures questions the routine use of solvent-tolerant *P. putida* host strains in future applications.

The successive oxidation of toluene to benzyl alcohol and benzaldehyde by XMO followed by the condensation with endogenous pyruvate to (\( R \))-phenylacetylcarbinol by pyruvate decarboxylase was evaluated in recombinant *E. coli*. Catalytic efficiency of this multistep reaction was found to significantly depend on the host metabolism in terms of gene expression, cofactor regeneration, and pyruvate synthesis. This biotransformation therefore represents a useful system for metabolic studies under reaction conditions.

This work shows that the selection of the host strain has direct impacts on the efficiency of biooxidations as well as of upstream and downstream processing and thus will certainly influence the design of future bioprocesses.
Zusammenfassung


Diese Arbeit zeigt, dass die Wahl des Wirtsstammes direkte Auswirkungen auf die Effizienz von Biooxidationen, das Upstream-Processing sowie auf die Produktaufarbeitung hat und beeinflusst daher das Design von zukünftigen Bioprozessen signifikant.
Chapter 1: *Introduction*

Daniel Meyer

Published in parts:


Process and catalyst design objectives for specific redox biocatalysis

1.1 General introduction

Microorganisms are employed as biocatalysts in the food and dairy sector and also in pharmaceutical and chemical industries (Ghisalba, 2000; Liese et al., 2000). An increasing number of pharmacologically active secondary metabolites are produced by microorganisms. Examples include antibiotics, steroids, or alkaloids for the treatment of infections, cancer, transplant rejection, and high cholesterol (Mijts and Schmidt-Dannert, 2003; Farnet and Zazopoulos, 2005). In the chemical industry, the recent trend towards environmental sustainability promotes the use of biomass (cellulose, starch, lignin, or sugars) as a renewable feedstock for the production of chemicals (Held et al., 2000; Parales et al., 2002; Werpy and Petersen, 2004). In a first step, fermentation products such as ethanol, acetic acid, butanol, lactic acid, or propanediol, can be obtained (Liese et al., 2000; Bommarius and Riebel, 2004; Ezeji et al., 2004; Lee et al., 2004; Vink et al., 2004; Bothast and Schlicher, 2005; Jeffries, 2005). These compounds as well as oil components can serve as substrates for the synthesis of high-value-added biotransformation products, for example, (chiral) fine and specialty chemicals, synths etc. (Bommarius and Riebel, 2004; Faber, 2004). Such biotransformations can be catalyzed by isolated enzymes as well as wild-type or recombinant microorganisms (over)producing the enzyme of interest (Sariaslan, 1989; Duetz et al., 2001; Schmid et al., 2001a; Matsuyama et al., 2002; Straathof et al., 2002; Ishige et al., 2005). The complex nature and the prevalent cofactor dependency of oxidoreductases constrict the use of isolated enzymes for redox biocatalysis (Bühler and Schmid, 2004; Faber, 2004). Thus, efforts towards the industrial application of oxidoreductases have mainly focused on whole-cell biocatalysis.

A crucial parameter in the development of a biocatalytic process is the productivity, which is defined by the yield of product per time and volume and influenced by the biocatalyst efficiency, by non-biological factors, such as the quality of raw materials or substrates (upstream processing), and by the efficiency of product removal and purification (downstream processing) (Fig. 1.1A). One important factor determining biocatalyst efficiency is its specific activity described as the specific rate of product formation during biotransformation, for example, in units (μmol product per minute) per gram of cell dry weight (CDW). It depends on the synthesis and activity of functional enzyme as well as on cofactor availability (Fig. 1.1A).
(A)

Productivity $[\text{g liter}^{-1} \text{ h}^{-1}]$ = $F(\text{Biocatalyst Activity and Stability, Upstream and Downstream Processing})$

Specific Biocatalyst Activity $[\text{Units/g CDW}]$ = $F(\text{Expression Level, Enzyme Activity, Cofactor Availability})$

(B)

Fig. 1.1. Factors determining process and biocatalyst efficiency.

Cell metabolism plays a key role by regenerating cofactors as donors or acceptors of reduction equivalents to support redox biocatalysis and for maintaining cell functionality, for example, resistance against toxic chemicals. Furthermore, not only the maximum performance but also the interaction and stability of cell metabolism, enzyme synthesis, and enzyme catalysis have to be considered for the evaluation of biocatalyst efficiency (Fig. 1.1B). Within this chapter, we discuss selected targets for intensification of redox bioprocesses (Fig. 1.2). To limit the scope, mass transfer of substrate/product across the cell envelope was omitted,
although the biotransformation rate may be increased by alterations in the outer membrane (van Beilen et al., 2003; Ni and Chen, 2004; Ni and Chen, 2005). Furthermore, aspects of enantio-selectivity in whole-cell biotransformations are not considered, although cell physiology may influence the enantio-specificity of a transformation (Bertau, 2002).

Here, we discuss gene expression, cell metabolism, cofactor availability, oxygen transfer, and catalyst stability as possible targets for improving catalyst efficiency (Fig. 1.2). Integrating these aspects on a molecular, physiological, and reaction-engineering level allows increasing the productivity of bioprocesses. In this respect, the selection of organic solvents and recombinant host strains is critically discussed. In particular, the applicability of solvent-tolerant bacteria in two-liquid-phase biotransformations is evaluated.

Fig. 1.2. Targets for bioprocess intensification.
1.2 Gene expression

The efficiency of a microbial biocatalyst depends on the amount of functional enzyme that is produced and maintained during biotransformation (Fig. 1.1B). Wild-type bacteria often have to be cultivated on specific substrates in order to induce the synthesis of catabolic enzymes used for biotransformation. Such enzymes are usually encoded as single gene copies on the bacterial genome or on catabolic plasmids, and are thus often synthesized only in relatively low amounts. Today, recombinant DNA technologies allow multiplying the gene templates, either by introducing further gene copies into the genome or by providing them on expression vectors. In addition, the level of gene expression can be fine-tuned by introducing and engineering of regulatory sequences such as promoters, enhancers, or ribosome-binding sequences.

1.2.1 Promoters

Various promoter sequences have been characterized and shown to be useful for the regulation of heterologous gene expression in Escherichia coli (Goldstein and Doi, 1995; Weickert et al., 1996). A strong promoter provides a high expression level, which results in high enzyme concentrations. However, high-level expression is not always desired, since it represents a metabolic burden for the cells by draining the resources needed for growth and productive reactions. High concentrations of “toxic” proteins may even be harmful for the cells (Goldstein and Doi, 1995). Host bacteria can respond to the overproduction of membrane-bound enzymes by increased phospholipid synthesis, which may have negative effects on the physiology and morphology of the cell (Nieboer et al., 1993; Chen et al., 1995a; Chen et al., 1996; Nieboer et al., 1996). Unproductive recombinant enzyme activity, such as the uncoupling observed for many oxidoreductases, may lead to a loss of cofactors and in the case of oxygenases to an increased oxygen demand and the production of toxic hydrogen peroxide (Loida and Sligar, 1993; Lee, 1999). The use of inducible promoters with tunable expression levels is, therefore, often preferred to constitutive gene expression for biotransformations with metabolically active cells.

The best studied promoter is that controlling the expression of the lac operon in E. coli. The lac system was among the first regulatory systems studied and is still frequently used as a tool in biotechnology, for example, in pUC-derived vectors (Gronenborn, 1976; Vieira and
Messing, 1982). However, the expression level reached via the lac promoter is relatively low. Derivatives of the lac promoter such as tac or trc, or T7 promoters provide higher expression levels but, as the lac promoter, show a relatively high basal expression in the absence of inducer (“leakiness”) (Weickert et al., 1996; Jonasson et al., 2002) (Table 1.1). Heterologous gene expression under the control of ara (or bad), rha, or alk promoter systems is “tighter” and can be finer tuned by titration of inducing agents (Guzman et al., 1995; Perez-Perez and Gutierrez, 1995; Haldimann et al., 1998; Panke et al., 1999b; Bühler et al., 2000). This was exploited in a number of biotransformations (Doig et al., 2002; Panke et al., 2002; Bühler et al., 2003b) and for the production of heterologous enzymes on a large scale (Doig et al., 2001; Hofstetter et al., 2004). Based on alk and ara promoters, vectors have been constructed that provide various selection markers and polylinkers for an optimized use in gene expression (Smits et al., 2001; Cronan, 2006). Another advantage of gene expression under the control of alk, ara, or rha promoter systems is the induction by relatively cheap chemicals, such as octane or dicyclopropyl ketone, arabinose, or rhamnose, respectively.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Inducer</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>lac</td>
<td>IPTG</td>
<td>Many vectors available (e.g., pUC derivatives)</td>
<td>Relatively low expression level; “leaky” expression; inducer rather expensive; repressed by glucose</td>
</tr>
<tr>
<td>tac or trc</td>
<td>IPTG</td>
<td>Many vectors available; high-level expression; induction level can be titrated</td>
<td>“Leaky” expression; inducer rather expensive</td>
</tr>
<tr>
<td>T7</td>
<td>IPTG</td>
<td>Many vectors available (e.g., pET derivatives); high-level expression</td>
<td>“Leaky” expression; difficult to achieve high cell densities; inducer rather expensive</td>
</tr>
<tr>
<td>alk</td>
<td>DCPK, octane etc.</td>
<td>High-level expression; induction level can be titrated; “tight” regulation; cheap inducer</td>
<td>Few vectors available</td>
</tr>
<tr>
<td>ara or rha</td>
<td>Arabinose or rhamnose</td>
<td>Wide range of induction levels possible; “tight” regulation; rapid induction/repression; cheap inducer</td>
<td>Few vectors available; catabolite repression by glucose</td>
</tr>
</tbody>
</table>

a Only induction by chemicals is considered; IPTG, isopropyl-β-D-thiogalactoside; DCPK, dicyclopropyl ketone.
In contrast, lac, tac, trc, and T7 promoters are induced by isopropyl-β-D-thiogalactoside (IPTG), which is not ideal due to rather high costs for IPTG. Lactose, the natural inducer of the lac operon, can be used as a cheap alternative for IPTG (Neubauer and Hofmann, 1994). However, its concentration and thus the expression level of the recombinant gene can change during biotransformation since it is metabolized by the cells. Yet, IPTG purchase prizes for quantities used in large-scale biotransformations are an order of magnitude lower than indicated in catalogues of chemical purchasers (Michael Schedel, Bayer HealthCare AG, Wuppertal, Germany, personal communication). In addition, already 50 to 100 μM IPTG can be sufficient for full induction (Baneyx, 1999). Thus, induction by IPTG is not necessarily a significant cost factor for the synthesis of high-value-added products on multi cubic meter scales.

Some promoter systems induce gene expression as a function of temperature, pH, or as a result of the depletion of a certain nutrient in the growth medium (Chou et al., 1995; Goldstein and Doi, 1995; Weickert et al., 1996). For biocatalysis, however, induction by the addition of a chemical is simpler than by its removal or by temperature or pH shifts. The optimization of reaction conditions, such as pH and temperature, generally aims at maximizing catalytic performance rather than gene expression.

On-going research with respect to promoter engineering and vector design will result in the development of new and improved expression systems with higher expression levels, reduced leakiness, and better regulation (Mertens et al., 1995a; Mertens et al., 1995b; Weickert et al., 1996; Alper et al., 2005; Royo et al., 2005). New expression vectors will be useful for more efficient syntheses of functional enzymes, and thus may lead to higher yields of value-added products in processes based on recombinant whole-cell biocatalysts.

1.2.2 Limitations of protein overproduction

Gene overexpression in a heterologous host does not always result in higher specific activities as compared to the native host. In other words, specific enzyme activities in recombinants are often lower than in the wild-type, which can be explained by differences in the molecular environment in different strains. Critical factors include genetic stability, transcriptional or translational regulation, RNA or protein stability, as well as protein folding, processing, and assembly (Zeyer et al., 1985; Zabriskie and Arcuri, 1986; Friehs and Reardon, 1993; Staijen et al., 1997; Staijen et al., 2000).
“Self-cloning”, the overexpression of the gene of interest in the native host, might be a suitable strategy to overcome problems associated with the strain specific expression machinery (Duetz et al., 2001). Broad host range vectors can be used for gene expression in bacteria different from E. coli (Mermod et al., 1986; Arai et al., 1991; Rangwala et al., 1991; Yen, 1991; Graupner and Wackernagel, 2000; Toukdarian, 2004). Since most of these vectors are present at low copy numbers, shuttle vectors with an increased plasmid copy number were constructed (Smits et al., 2001; Tao et al., 2005). These plasmids are useful for gene expression in various hosts, but their suitability for biocatalysis under large-scale process conditions, including aspects such as genetic and segregational stability, has hardly been investigated so far.

Stable biocatalysts have been constructed by placing genes from Pseudomonas strains via transposons into the chromosome of strains belonging to the same genus or species (Panke et al., 1999a; Wery et al., 2000; Hüsken et al., 2001a; Wierckx et al., 2005). Although only one set of styrene monooxygenase genes (styAB) from Pseudomonas sp. strain VLB120 was introduced into the genome of Pseudomonas putida KT2440, the specific styrene oxidation rate of engineered P. putida SAB was higher than the rate obtained with recombinant E. coli JM101 (pSPZ10) overexpressing the genes on a multi copy number plasmid (Panke et al., 1999a). In contrast, styrene monooxygenase activity was not detectable in E. coli strains engineered in the same way, and harboring one set of styAB genes on the chromosome.

In another study, the potential drawback of low enzyme concentrations due to a single chromosomal gene insertion was addressed by placing up to 150 copies of mercury resistance and toluene dioxygenase genes in the genome of Deinococcus radiodurans, the most radiation-resistant organism known (Brim et al., 2000). The resulting engineered bacterium can be employed for bioremediation of radioactive wastes containing ionic mercury and toluene.

Metagenome screening suggests that only a minor part of identified genes can be expressed in E. coli hosts (Gabor et al., 2004; de Lorenzo, 2005). Thus, recombinants based on host strains providing appropriate molecular environments for gene expression will be essential to exploit newly identified enzyme activities for biocatalysis. Yet, further developments of molecular biological tools and an in-depth physiological characterization are required to make alternative host strains accessible for recombinant biocatalysis. Here, the PlugBug concept of DSM (Heerlen, The Netherlands) represents a promising approach, which makes use of only a limited number of different, well characterized host strains for the synthesis of the majority of biocatalytically produced DSM products.
1.3 Cell metabolism

Residual substrates or side-products frequently complicate downstream processing because of their similar physiochemical properties as compared to the product. Substrates and products can be either chemically, thermally, or biologically unstable under reaction conditions leading to side-product formation and product degradation. Biological instability may be caused by the metabolic machinery of the microbial host leading to uncontrolled substrate or product conversion by host enzymes.

Normally, bacteria are cultivated in aqueous, often aerobic environments with temperature and pH kept within physiological ranges. Biocatalytic production of compounds that are chemically unstable under these conditions, such as epoxides (Panke et al., 2000; Hofstetter et al., 2004) or catechols (Held et al., 1999; Wery et al., 2000; Meyer et al., 2003), requires suitable reaction-engineering solutions such as the in situ extraction of the reactants from the cell broth with a protective organic phase (Lye and Woodley, 1999). This organic phase may either be a liquid or a solid, which stabilizes substrate and product as well as protects cells from toxic effects (see Section 1.6.1).

On the one hand, catabolic pathways of bacteria able to metabolize a broad range of aromatic chemicals are interesting for biocatalytic applications (Jimenez et al., 2002; Wackett, 2003). An example is the use of *P. putida* for the cometabolic production of a variety of heteroaromatic carboxylic acids on cubic meter scale by Lonza AG (Visp, Switzerland) (Kiener, 1992; Schmid et al., 2001a). On the other hand, the catabolic potential of such microbial strains can result in side-product formation or breakdown of substrate/product during biocatalysis.

The inactivation of disturbing enzyme activities or the cloning of genes encoding the enzymes of interest in microbial hosts, which provide a “neutral” enzymatic background are strategies to avoid biological product instability.

1.3.1 Avoidance of side-product formation

Additives or inhibitors may be used to reduce enzymatic side-product formation, but in bioprocesses, they are seldom applied. Reasons may be higher costs or the associated increase in complexity of such a process (Bühler and Schmid, 2004). Nevertheless, this approach is well documented for the production of phenylacetylcarbinol from pyruvate and benzaldehyde...
by pyruvate decarboxylase during yeast fermentation (Oliver et al., 1999), which is one of the earliest biotechnological processes relevant for the chemical industry (Liese et al., 2000). Significant amounts of benzaldehyde are lost as substrate for pyruvate decarboxylase due to the activity of alcohol dehydrogenases, which reduce benzaldehyde to benzyl alcohol (Nikolova and Ward, 1991). The most effective method to prevent the formation of benzyl alcohol from benzaldehyde during phenylacetylcarbinol production is the addition of alternative proton acceptors beside benzaldehyde, such as colloidal sulfur or acetaldehyde. However, due to the high toxicity of sulfur, only acetaldehyde was applied in fermentative phenylacetylcarbinol production. Conversion of benzaldehyde to phenylacetylcarbinol increased from 40% to 70% accompanied by a proportional decrease of benzyl alcohol formation (Smith and Hendlin, 1954; Oliver et al., 1999). The use of nicotinic acid analogues to compete with NADH for enzyme active sites or iodoacetic acid as an inhibitor of sulfhydryl-containing enzymes (such as alcohol dehydrogenases) brought only little success since these compounds inhibited metabolically important enzymes. It is noteworthy that cell-free bioprocesses yield higher phenylacetylcarbinol productivities since no reduction of benzaldehyde to benzyl alcohol occurs when cell-free extracts or purified pyruvate decarboxylase are used as biocatalysts (Rosche et al., 2002).

The use of mutants without interfering side-activities is another possibility to reduce enzymatic substrate/product degradation during biotransformation (Knop et al., 2001). This method enables the design of biocatalysts, which accumulate an intermediate product as a result of an interrupted metabolic pathway (Bosetti et al., 1992). As an example, an E. coli mutant was generated, which is completely blocked in its ability to metabolize pyruvate as a result of the deletion of genes coding for pyruvate dehydrogenase, pyruvate formate lyase, pyruvate oxidase, PEP synthetase, and lactate dehydrogenase (Zelic et al., 2003). Glucose was converted to pyruvate, which accumulated to concentrations higher than 62 g/liter in fed-batch cultivations with acetic acid as sole carbon source. However, genetic engineering can be complicated if substrate and/or product degradation is catalyzed by multiple enzyme activities and enzymes with relaxed substrate specificities, for example, dehydrogenases. Comparative analysis of the P. putida KT2440 genome revealed up to 40 putative dehydrogenases, of which the substrate specificity is unknown (Nelson et al., 2002). Thus, the generation of mutants, which are not able to degrade a certain substrate/product, might require multiple deletions of unspecific enzyme activities. This raises the question whether such genetically engineered biocatalysts still provide the metabolic fitness to efficiently catalyze energy-dependent redox reactions and to cope with harsh process conditions.
Introduction

1.3.2 The use of recombinant host strains

Bacteria expressing heterologous genes are applied for the biocatalytic production of a broad range of value-added chemicals. Next to bacteria, also yeast species are used as recombinant biocatalysts and are of particular interest for glycosylations (Wohlgemuth, 2005). Recombinant whole-cell biocatalysis has developed to a standard technology, which frequently uses *E. coli* strains as bacterial hosts (Schmid *et al.*, 2001a). The high popularity of this host bacterium may be due to its excellent accessibility by molecular biology methods, high achievable growth rates and biomass yields on minimal medium with cheap carbon sources, non-pathogenicity, and its high cofactor regeneration potential. In addition, the PCR technology allows accessing the genetic information of currently more than 300 complete bacterial genomes (http://www.tigr.org/), which can be expressed in *E. coli* by numerous compatible gene expression systems (Yasueda and Matsui, 1994; Baneyx, 1999).

*E. coli* is mainly known as an inhabitant of the intestines of warm-blooded animals, where it is not expected to get in touch with a broad range of, for example, aromatic compounds. Thus, *E. coli* strains are often assumed to have only a low tendency to degrade aromatic substrates and products and to provide a “neutral” enzymatic background activity in recombinant biocatalysis. However, Diaz *et al.* summarized *E. coli* catalyzed reactions involved in the degradation of many aromatic compounds, which illustrates that this bacterium is not a catabolically “empty box” and, therefore, is not a universally applicable host strain for all kinds of biotransformations (Diaz *et al.*, 2001). (Multiple) knockout mutations to reduce or delete interfering enzyme activities, however, can threaten metabolic activity of the cells, which is a prerequisite for maintaining general metabolic function, cofactor regeneration, and continuous synthesis of recombinant enzymes. The systematic screening and classification of microorganisms with an enzymatic background activity that is suitable for catalysis of a desired reaction type thus represents a promising strategy to overcome substrate and product degradation.
1.4 Cofactor availability

Most oxidoreductases are cofactor dependent and reactions catalyzed by such enzymes are usually associated with the consumption of stoichiometric amounts of NAD(P)/H (Harayama et al., 1992). In order to make biotransformations with isolated enzymes or preparations from crude extracts economically feasible, enzymatic or non-enzymatic cofactor regeneration systems are required (Faber, 2004; Hollmann and Schmid, 2004; Wichmann and Vasic-Racki, 2005). Whole-cell biotransformations generally do not require additional regeneration systems, since cofactors are regenerated by the metabolic activity of the microbial catalyst. To make such whole-cell processes interesting as alternatives to classical organic syntheses, high productivities are required (Schmid et al., 2001a). This raises the question about a possible limitation of whole-cell redox biocatalysis by the cofactor regeneration capacity of the cell metabolism.

Seven different bacterial species cultivated aerobically in batch mode at 30°C were recently investigated for pathways involved in glucose metabolism (Fuhrer et al., 2005). In order to evaluate the potential of these strains to serve as hosts for NAD(P)H-dependent redox biocatalysis, we used the reported growth physiological data to estimate their reductive cofactor regeneration capacity (Table 1.2). For the calculation, the elemental ratio of biomass was assumed to be CH_{1.67}O_{0.27}N_{0.2} (Duetz et al., 2001). In order to simplify the estimation, every molecule of glucose not metabolized for biomass or acetic acid production was assumed to lead to the generation of eight molecules of NADH and two molecules of NADPH. Every molecule of accumulated acetic acid was assumed to result in the generation of two molecules of NADH. However, cofactor specificity of enzymes involved in NAD(P)H generation may vary among different species. Since transhydrogenases can convert NADPH to NADH and vice versa by transferring electrons directly from NADPH to NAD^+ or from NADH to NADP^+ (Jackson, 2003; Sauer et al., 2004), the total pool of oxidized nicotinamid cofactors may be available, for example, for NADH-dependent biocatalysis. Resulting (total) NAD(P)H regeneration rates during growth of the different bacterial species were between 126 and 2218 U/g CDW (Table 1.2). This is in accordance with earlier calculations, which proposed a rate of 720 U/g CDW for NADH regeneration (Duetz et al., 2001). The NAD(P)H regeneration rate of E. coli was estimated to be about 575 U/g CDW, which is in the same order of magnitude as the maximum specific activities of oxygenases that have been reached in practice (about 500 U/g CDW; (Duetz et al., 2001)). Under the assumption that non-
growing cells have the same capacity for glucose catabolism, the glucose that is metabolized for biomass formation would become available for cofactor regeneration (Table 1.2). As a result, the estimated NAD(P)H regeneration rate for non-growing *E. coli* cells increases to 1125 U/g CDW. *Paracoccus versutus*, a facultative autotroph belonging to the *α*-proteobacteria, showed the highest specific glucose uptake rate during growth, which resulted in about three- to four-fold higher estimated NAD(P)H regeneration rates than for *E. coli* (Table 1.2). Although this species is not yet exploited for biocatalytic applications, its enormous TCA cycle activity makes it interesting as an alternative host for future applications with high demands for cofactor regeneration. In spite of *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, and *Rhodobacter sphaeroides* showed a lower NAD(P)H regeneration potential, other *α*-proteobacteria might be found with potentials comparable to *Paracoccus versutus*, which might promote the use of strains, such as *Sphingomonas* sp., as biocatalysts for oxidative biotransformations (Li *et al.*, 2001).

The simplified calculation of NAD(P)H regeneration rates considers neither maintenance energy demands nor energy consumption as a result of stress responses, which may appear under bioprocess conditions. High concentrations of substrates, products, or organic solvents in the cell broth can have toxic effects with membrane disintegration being the most pronounced mechanism of solvent toxicity (Sikkema *et al.*, 1995) (see Section 1.6). Thus, uncoupling of the proton motive force eventually reduces the amount of cofactors available for biocatalysis. A recent study on stereospecific styrene epoxidation catalyzed by recombinant *E. coli* containing styrene monoxygenase from *Pseudomonas* sp. strain VLB120 suggests that a product toxicity induced cofactor limitation affected the styrene oxide productivity under process conditions (Park *et al.*, 2006).

Uncoupling of energy demands for biotransformation from demands for cell growth is an interesting issue in biocatalysis. Unfortunately, many processes involving oxygenases are only productive when growing cells are used as biocatalysts (Favre-Bulle *et al.*, 1991; Favre-Bulle and Witholt, 1992; Favre-Bulle *et al.*, 1993; Bühler *et al.*, 2003a; Bühler *et al.*, 2003b). Possible reasons include decreasing intracellular oxygenase concentrations due to changes in the regulation of gene expression (Ishihama, 1997), protein stability (Gottesman, 2003), or cell metabolism and physiology (membrane stability). A decreasing metabolic activity may affect NAD(P)H regeneration when cells enter the stationary growth phase. Here, metabolic engineering may contribute to a better understanding and enable the manipulation of metabolic fluxes in order to channel energy to biocatalysis instead of biomass synthesis (Lee *et al.*, 2003; Wandrey, 2004; Poulsen *et al.*, 2005).
Table 1.2. Growth physiology and NAD(P)H regeneration rates of different bacterial strains cultivated aerobically on glucose in batch-mode

<table>
<thead>
<tr>
<th>Organism</th>
<th>Max. spec. growth rate</th>
<th>Biomass yield</th>
<th>Glucose uptake rate</th>
<th>Acetic acid accum. rate</th>
<th>Estimated cofactor regeneration rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[h⁻¹]</td>
<td>[g CDW/g gluc.]</td>
<td>[mmol g⁻¹ h⁻¹]</td>
<td>[mmol g⁻¹ h⁻¹]</td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>0.49</td>
<td>0.44</td>
<td>4.5</td>
<td>13.7</td>
<td>36.0</td>
</tr>
<tr>
<td>S. meliloti</td>
<td>0.17</td>
<td>0.41</td>
<td>2.3</td>
<td>7.8</td>
<td>18.4</td>
</tr>
<tr>
<td>A. tumefaciens</td>
<td>0.3</td>
<td>0.41</td>
<td>4.1</td>
<td>13.8</td>
<td>32.8</td>
</tr>
<tr>
<td>Paracoccus versutus</td>
<td>0.70</td>
<td>0.21</td>
<td>18.9</td>
<td>106.4</td>
<td>151.2</td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>0.15</td>
<td>0.41</td>
<td>1.8</td>
<td>6.1</td>
<td>14.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.39</td>
<td>0.30</td>
<td>7.8</td>
<td>29.0</td>
<td>55.4</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>0.30</td>
<td>0.35</td>
<td>4.8</td>
<td>15.3</td>
<td>34.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>NADH</th>
<th>NADPH</th>
<th>NADH</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[mmol g⁻¹ h⁻¹]</td>
<td>[U g⁻¹ h⁻¹]</td>
<td>[mmol g⁻¹ h⁻¹]</td>
<td>[U g⁻¹ h⁻¹]</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>13.7</td>
<td>228</td>
<td>3.4</td>
<td>57</td>
</tr>
<tr>
<td>S. meliloti</td>
<td>7.8</td>
<td>129</td>
<td>1.9</td>
<td>32</td>
</tr>
<tr>
<td>A. tumefaciens</td>
<td>13.8</td>
<td>231</td>
<td>3.5</td>
<td>58</td>
</tr>
<tr>
<td>Paracoccus versutus</td>
<td>106.4</td>
<td>1774</td>
<td>26.7</td>
<td>444</td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>6.1</td>
<td>101</td>
<td>1.5</td>
<td>25</td>
</tr>
<tr>
<td>E. coli</td>
<td>29.0</td>
<td>483</td>
<td>5.5</td>
<td>92</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>15.3</td>
<td>254</td>
<td>2.8</td>
<td>46</td>
</tr>
</tbody>
</table>


Data obtained from (Fuhrer et al., 2005).

See text for details.

One unit is defined as one µmol NAD(P)H regenerated per minute.
Another factor which reduces cofactor availability is the interference of enzymatic background activity with biocatalysis. Such an interference was observed, when recombinant E. coli containing xylene monooxygenase were used for the successive oxidation of toluene and xylenes to the corresponding benzyl alcohols, benzaldehydes, and benzoic acids (Bühler et al., 2000; Maruyama et al., 2003). In these applications, the oxidation of alcohols to the corresponding aldehydes catalyzed by the monooxygenase was counteracted by the reduction of aldehydes to alcohols catalyzed by dehydrogenases from the E. coli host. This “futile cycle” not only reduced the aldehyde formation rate but also acted as a sink for reduced cofactors, since both reactions use stoichiometric amounts of NADH (Harayama et al., 1992; Bühler et al., 2000). On the other hand, the involvement of both, oxygenases and dehydrogenases for the catalysis of successive oxidations uncouples biocatalysis from cofactor regeneration by the host metabolism. The NADH consumption in the oxygenase-catalyzed reaction, thereby, is compensated by NADH generation in the dehydrogenase-catalyzed oxidation. This approach was used in an environmental application for the biodegradation of 2-chlorotoluene by engineered pseudomonads (Haro and de Lorenzo, 2001) and in synthetic applications for the production of 3-methylcatechol from toluene by genetically engineered P. putida strains (Wery et al., 2000; Hüsken et al., 2001a).

Overall, specific activities do not seem to be limited by cofactor availability in current industrial processes. However, this issue will certainly be of importance in future applications using optimized biocatalysts with higher specific activities or in applications running under conditions, which impose an additional burden on the metabolism of the microorganisms involved.

1.5 Oxygen transfer

High oxygen transfer rates are important for all aerobic bioprocesses and crucial for biocatalytic reactions with molecular oxygen as a substrate. Oxygenases, for instance, incorporate molecular oxygen into a second substrate. In whole-cell biotransformations, oxygen has to be transferred from air to the enzyme via the cultivation medium and the cell envelope. The oxygen solubility is relatively low in water, which can lead to oxygen limitation in oxygenase-based biotransformations involving high concentrations of (respiring) cells, because generally respective K_m values of oxygenases are significantly higher than those of the cytochrome complexes in the electron-transfer chain (Duetz et al., 2001).
Technically, oxygen transfer can be improved by increasing the stirring rate (which results in the formation of smaller air bubbles), the oxygen content in the gas influx, or the pressure inside the reactor (Onken and Liefke, 1989; Schmid et al., 1999), or by the addition of a second liquid phase with a higher oxygen solubility than water (oxygen vectors) (Rols and Goma, 1989; Rols et al., 1990; Giridhar and Srivastava, 2000). Nevertheless, especially in large-scale applications, oxygen transfer can become limiting for cell growth and/or biocatalysis. In a stirred-tank reactor on a laboratory scale aerated with air, maximum oxygen transfer rates in the range of 300 – 400 mM h\(^{-1}\) have been reached. Industrial, 100-cubic meter stirred-tank reactors show significantly lower oxygen transfer rates, which are typically below 100 mM h\(^{-1}\) (Schmid, 1997; Held et al., 2000).

Uncoupling of oxygen demands used for respiration during growth from demands for oxygenase catalysis might be achieved by biotransformation in the stationary phase or in recombinant hosts with low endogenous respiration activities, which also may influence NAD(P)H availability. Here, the question is to what extent NAD(P)H regeneration is driven by demand, especially in resting cells (see Section 1.4).

An interesting approach for enhanced oxygen supply is the addition of hydrogen peroxide to microbial cultures in the presence of catalase, which converts hydrogen peroxide to water and molecular oxygen (Schlegel, 1977; Nies and Schlegel, 1984). Catalase is synthesized by many microorganisms or it can be added separately to the culture. Using this approach, cells incubated in an anaerobic nitrogen atmosphere grew at the identical rates as cells incubated under standard aerobic conditions. Specific activities of endogenous catalase for the cleavage of hydrogen peroxide are relatively high (e.g., \textit{P. putida}, 50'000 U/g CDW (Schlegel, 1977)). The fast turnover of toxic hydrogen peroxide might therefore explain the absence of growth inhibition. The addition of hydrogen peroxide can be regulated as a function of dissolved oxygen tension in the culture broth or of oxygen partial pressure in the off-gas of the bioreactor (Sonnleitner and Hahnemann, 1997). This setup was useful for the production of gluconic acid from glucose by \textit{Aspergillus niger} (Rosenberg et al., 1992) and might be especially suitable for biotransformations requiring low aeration rates to minimize the loss of volatile reactants, foam formation, and explosion hazard (Sonnleitner and Hahnemann, 1997). Oxygen transfer to cells susceptible to hydrodynamic stresses can be performed by bubble-free aeration across membranes (Lütz et al., 2005). Application of hydrogen peroxide and catalase thereby may represent an interesting alternative, although the hydrogen peroxide sensitivity of, e.g., mammalian cells may preclude such a strategy.
In contrast to oxygenase-catalyzed reactions, hydroxylations catalyzed by peroxidases and dehydrogenases do not require molecular oxygen as a cosubstrate, thus turning them into particularly interesting candidates for the catalysis of hydroxylations. Peroxidases catalyze a large variety of hydroxylations and thereby produce one molecule of water from hydrogen peroxide (or alcohol in the case of organic peroxide driven peroxidations) as a coproduct (van Deurzen et al., 1997b; Adam et al., 1999; van Rantwijk and Sheldon, 2000). A further advantage of peroxidases is that they need no regeneration of cofactors such as NAD(P)H. However, a major shortcoming is the low operational stability of peroxidases, generally resulting from peroxide induced deactivation (van de Velde et al., 2001). An example is the facile oxidative deterioration of the porphyrin ring in heme-dependent peroxidases such as the frequently used chloroperoxidase (van Deurzen et al., 1997a; van de Velde et al., 2000).

Dehydrogenases can incorporate oxygen derived from water. As an example, the bacterial hydroxylation of 6-methylnicotinic acid to 2-hydroxy-6-methylnicotinic acid was performed under aerobic but oxygen-limited conditions (Tinschert et al., 1997). Hydroxylations of a variety of \(N\)-heteroaromatic carboxylic acids have been described (Hille, 1996; Kaiser et al., 1996; Tinschert et al., 1997; Wieser et al., 1997; Fetzner, 1998; Fetzner, 2000) and were exploited for synthetic applications in industry (Kulla, 1991; Schmid et al., 2001a). However, it is noteworthy that molecular oxygen is one proposed electron acceptor in such dehydrogenase-catalyzed reactions, which might impair or prohibit the uncoupling of biooxidations from oxygen transfer rates (Stephan et al., 1996).

1.6 Catalyst stability and inactivation

The productivity of a process is significantly influenced by the stability and robustness of the microbial biocatalyst under reaction conditions (Fig. 1.1B). The toxicity of substrates, products, or organic solvents involved in biocatalysis thereby plays a major role. The integration of organic chemicals (also referred to as organic solvents) in the lipid bilayer impairs structure and function of the membrane and ultimately leads to cell death (Sikkema et al., 1994; Sikkema et al., 1995). The tendency of organic solvents to partition into membranes was found to correlate with their partitioning into the octanol phase of an octanol–water mixture (Seeman, 1972; Sikkema et al., 1995). The logarithm of the octanol–water partition coefficient (\(\log P_{ow}\)) is therefore a useful measure for the assessment of cell-toxicity of organic solvents (Laane et al., 1987). It was found that the toxicity tends to increase with a decreasing
logPow. A solvent with a logPow below 4 is generally considered to prevent growth of non-solvent-tolerant strains. Although organic solvents with a logPow over 4 partition better into membranes than low-logPow solvents, their aqueous solubility is not high enough to cause lethal membrane concentrations (Osborne et al., 1990; Weber and de Bont, 1996; de Bont, 1998). Some chemicals provoke additional toxic effects, which are independent from the tendency to accumulate in membranes (Rajagopal, 1996). As an example, nitro or amine groups render aromatic chemicals more toxic for microorganisms than predicted based on their hydrophobicity (Donlon et al., 1995). Next to this molecular toxicity, cells can be affected by the presence of a second liquid phase of an organic solvent (phase toxicity), e.g., via a reduced substrate mass-transfer from the aqueous phase to the cell or by extraction of essential nutrients from the cell broth or membrane components from the cell envelope into the organic phase (Bar, 1986; Hocknull and Lilly, 1988).

A wide range of cytotoxic chemicals, such as phenol, catechol, toluene, styrene, xylenes, and derivatives thereof are highly interesting starting compounds in redox biocatalysis. Reaction engineering on the one hand and the use of more robust biocatalysts on the other hand are approaches, which make these substrates accessible for whole-cell biocatalysis.

1.6.1 Two-liquid-phase biotransformation

In order to handle the toxicity of organic low-logPow reactants during biotransformation, a second, water-immiscible, extractive phase can be introduced, which consists either of a solid or a liquid. Solid phases, for example, hydrophobic resins or capsules with a hydrophobic core, can be used for continuous product extraction, thereby effectively maintaining product concentrations in the cell broth at subtoxic levels (Wang et al., 1981; Voser, 1982; Hecht et al., 1987; Nielsen et al., 1988; Vicenzi et al., 1997; Held et al., 1999; Lye and Woodley, 1999; Meyer et al., 2003; Stark et al., 2003). Ideally, second liquid phases serve as a sink for products and as a substrate reservoir. Thus, high concentrations of cytotoxic substrates and products can be present in emulsions consisting of a high-logPow organic solvent and an aqueous medium. This method not only minimizes toxic effects for the cells in the aqueous phase, but can also reduce substrate/product inhibition of biocatalytically relevant enzymes, favorably shift the reaction equilibrium towards product formation, or increase oxygen transfer to the cells (Brink and Tramper, 1985; Rols and Goma, 1989; Leon et al., 1998). Two-liquid-phase biotransformation setups with dioctylphthalate (logPow, 9.6) or medium- to
long-chain alkanes, such as hexadecane (logP_{ow}, 8.8) or octane (logP_{ow}, 4.5), as second phases were exploited for selective biooxidations resulting in a wide number of products, including aromatic or aliphatic epoxides, alcohols, aldehydes, and acids as well as dihydrodiols and catechols (Bühler and Schmid, 2004). The production of 1-octanol from octane in an integrated two-liquid-phase bioprocess was evaluated with respect to reactor design and product recovery (Mathys et al., 1999).

The extraction potential of very hydrophobic organic solvents is reduced for a number of low-logP_{ow} chemicals. In a hexadecane–water mixture, chemicals such as phenol, cresol, or benzyl alcohol, partition preferably to water, whereas in octanol–water they partition preferably to octanol (Table 1.3 adapted from (Abraham et al., 1994)). Thus, for the production of these chemicals, the use of organic solvents with a relatively low logP_{ow}, such as octanol, could be more efficient as compared to hexadecane or dioctylphthalate. Such low-logP_{ow} solvents, however, are toxic to “normal” bacteria. A two-liquid-phase biotransformation system based on octanol as a second phase thus requires bacteria that can withstand these extreme conditions (see Section 1.6.2).

How can the organic solvent with the best extraction properties for a certain chemical be identified? Partition coefficients of a variety of chemicals in different organic solvent–water mixtures represent one useful source to estimate the suitability of an organic solvent as a second phase for the extraction of (groups of) chemicals (Abraham et al., 1994). The logP_{ow} values of octanol and cyclohexane are 3.1 and 3.4, respectively, and thus quite similar. However, the extraction properties of cyclohexane for compounds, such as phenol or benzyl alcohol, are more comparable to the properties of hexadecane (logP_{ow}, 8.8) than to those of octanol, which may be explained by the nonpolar character of cyclohexane (Table 1.3). This shows that partitioning behavior is not only determined by the logP_{ow} but also is influenced by hydrogen-bond formation, molecule size, and polarity of both solvent and solute (Abraham et al., 1994). Solutes with carbonyl substituents have often similar partition coefficients in mixtures with octanol, cyclohexane, and hexadecane, whereas the corresponding partition coefficients of solutes with hydroxyl and carboxyl groups are different. Also the position of substitutions can have a significant influence. As an example, the nitrophenol isomers have similar partition coefficients in an octanol–water mixture, whereas in a hexadecane–water mixture, the aqueous 2-nitrophenol concentration is about 700- and 2300-fold lower than the respective aqueous 3-nitrophenol and 4-nitrophenol concentrations (Abraham et al., 1994).
Table 1.3. Partition coefficients of chemicals in two-liquid-phase systems consisting of water and different organic solvents (adapted from (Abraham et al., 1994))

<table>
<thead>
<tr>
<th>Solute</th>
<th>Octanol–water (logP&lt;sub&gt;ow&lt;/sub&gt;, 3.1)</th>
<th>Cyclohexane–water (logP&lt;sub&gt;ow&lt;/sub&gt;, 3.4)</th>
<th>Hexadecane–water (logP&lt;sub&gt;ow&lt;/sub&gt;, 8.8&lt;sup&gt;b&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane</td>
<td>3.3</td>
<td>4.1</td>
<td>3.8</td>
</tr>
<tr>
<td>o-Xylene</td>
<td>3.1</td>
<td>n. a.</td>
<td>3.3</td>
</tr>
<tr>
<td>Styrene</td>
<td>3.0</td>
<td>n. a.</td>
<td>3.0</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.7</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>2.0</td>
<td>−0.24</td>
<td>−0.23</td>
</tr>
<tr>
<td>Benzene acid</td>
<td>1.8</td>
<td>−0.78</td>
<td>n. a.</td>
</tr>
<tr>
<td>Phenol</td>
<td>1.5</td>
<td>−0.85</td>
<td>−1.0</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1.5</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>1.2</td>
<td>−0.54</td>
<td>−0.53</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.9</td>
<td>−2.7</td>
<td>n. a.</td>
</tr>
</tbody>
</table>

n. a.: not available.

<sup>a</sup> LogP values are means of observed and calculated partition coefficients given by (Abraham et al., 1994).

<sup>b</sup> Data from (Laane et al., 1987).

In addition, styrene and styrene oxide are more efficiently extracted by dioctylphthalate as a second liquid phase than by hexadecane (Panke et al., 2000), which can be explained by the chemical similarity (aromaticity) of the solutes and dioctylphthalate. It is evident that partition coefficients of chemicals determined in an organic solvent–water mixture do not necessarily represent their partitioning behavior observed in an organic solvent–medium mixture (Hüsken et al., 2001b). Differences between theoretical estimations and reality may be even more pronounced for partitioning in an organic solvent–medium–cells mixture.

With regard to practical applications, further aspects beside extraction properties of an organic solvent have to be taken into account. Organic solvents can provoke many undefined physical phenomena with undesired side effects such as biomass clotting, cell aggregation at the liquid–liquid interface, loss of catalytic activity (via, e.g., enzyme inhibition), medium component accumulation in the organic phase, and slow coalescence (Brink and Tramper, 1985; van Sonsbeek et al., 1993; Schmid et al., 1998; Bertau and Scheller, 2003). These effects might be enforced or diminished depending on the strain used as a biocatalyst, for example, bacteria producing surface active compounds may provoke the formation of stable emulsions (Cooper and Zajic, 1980; de Smet et al., 1983). Biomass clotting and aggregation or solvent emulsification and dispersion can complicate the determination of biomass concentration by spectrophotometric means during a two-liquid-phase biotransformation.
(Aono et al., 2001; Hüsken et al., 2001b). The biomass concentration represents a key parameter for the description of whole-cell processes having a direct influence on the determination of process parameters such as specific substrate uptake, product formation, or growth rates (Sonnleitner et al., 1992). Gravimetric determination of CDW, counting of colony forming units, or determination of total protein content do not allow monitoring of biomass concentration “on-line” or “at-line” during biocatalysis, which is critical for process regulation. To circumvent these problems, cell growth is often followed by measuring the dissolved oxygen tension in the cell broth or the carbon dioxide/oxygen content in the off-gas (Hüsken et al., 2001b; Neumann et al., 2005; Wierckx et al., 2005). However, these parameters might be influenced by stress responses of the bacteria, especially when toxic reactants are involved. In addition, a changing surface tension, foam formation, or high viscosity of the emulsion may influence analytical instruments. Furthermore, the extraction solvent should be chemically, biologically, and thermally stable, available in bulk amounts at low prices, and nonhazardous for both operator and environment (Daugulis, 1988; Bruce and Daugulis, 1991).

In general, the evaluation of the suitability of second liquid phases by comparing partition coefficients simplifies the selection of an appropriate organic solvent with respect to biocompatibility and extractive properties. Computer-assisted screening methods were established that can help to identify the optimal solvent (Kollerup and Daugulis, 1985). However, these predictions do not replace the ultimate experimental confirmation of the suitability of a solvent or a bacterial strain for a certain two-liquid-phase biotransformation setup (Rojas et al., 2004; Neumann et al., 2005).

1.6.2 Application of solvent-tolerant bacteria in two-liquid-phase biotransformations

In two-liquid-phase biotransformations, the extraction properties of organic solvents like toluene or octanol are favorable for the production of chemicals with intermediate polarity such as catechols (see Section 1.6.1). However, “normal” bacteria are toxified by such organic solvents but can be substituted by extremophilic bacteria which can tolerate low-logPow organic solvents (de Bont, 1998).
1.6.2.1 **Mechanisms of solvent tolerance**

During the past decades, an increasing number of solvent-tolerant bacteria have been discovered (Ramos et al., 2002; Sardessai and Bhosle, 2002). Recently, also archaea (Usami et al., 2003; Usami et al., 2005) and eukaryotic microorganisms, such as yeast (Kawamoto et al., 2001) or algae (McEvoy et al., 2004), were reported to be tolerant to toxic organic solvents. A comparison among different bacterial species revealed that Gram-negative strains of the genera *Pseudomonas*, *Escherichia*, *Serratia*, and *Klebsiella* generally support the presence of organic solvents with lower logP<sub>ow</sub> than Gram-positive strains (Inoue and Horikoshi, 1991; Rajagopal, 1996). However, Gram-positive bacteria with solvent tolerance comparable to Gram-negative strains were recently characterized (Matsumoto et al., 2002; Na et al., 2005; Nielsen et al., 2005). The majority of solvent-tolerant bacteria isolated so far belongs to the genus *Pseudomonas*. Strains of this genus have been reported to be tolerant to hexane, cyclohexane, p-xylene, styrene, octanol, heptanol, toluene, or dimethylphthalate (Ramos et al., 2002; Segura et al., 2003; Sardessai and Bhosle, 2004; Wery and de Bont, 2004; Faizal et al., 2005; Wierckx et al., 2005).

In general, Gram-negative bacteria are considered solvent-tolerant if they can grow in the presence of an organic phase with a logP<sub>ow</sub> below 4, representing conditions that are lethal for “normal” bacteria. Alternatively, solvent tolerance is evaluated via the survival of bacteria after a short-term exposure to organic solvents (Ramos et al., 1995; Ramos et al., 1998).

Molecular and physiological mechanisms of solvent tolerance were investigated and characterized best in *P. putida* strains, namely *P. putida* DOT-T1E (Ramos et al., 1995) and *P. putida* S12 (Hartmans et al., 1990). Tolerant strains were found to differ from sensitive strains in the ability to activate protection mechanisms in the cell envelope upon incubation with toxic solvents. Alterations of membrane lipids to make the cell envelope more rigid and active efflux of solvent molecules into the extracellular medium are the proposed microbial strategies to cope with toxic solvents (Ramos et al., 2002). Mutants derived from solvent-tolerant *P. putida* DOT-T1E that are either deficient in cis-trans isomerases (Junker and Ramos, 1999) or solvent efflux pumps (Rojas et al., 2001) confirmed the impact of the respective mechanisms by being significantly more sensitive to organic solvents. These observations indicate that both isomerase and efflux pumps are required for efficient solvent tolerance.
Cis-trans isomerases convert cis-unsaturated fatty acids of membrane phospholipids to the trans-isomers as a major short term response to solvent exposure (Heipieper et al., 1992; Weber et al., 1994; Holtwick et al., 1997; Junker and Ramos, 1999). Other alterations of the membrane composition, such as changes in the degree of saturation of fatty acids, modifications of phospholipid head groups, and an increase in the total amount of phospholipids, were also observed as a result to solvent exposure (Weber and de Bont, 1996; Pinkart and White, 1997; Ramos et al., 1997; Segura et al., 2004). The resulting, more compact packing of the altered phospholipids in the lipid bilayer increases the rigidity of the cell envelope, thus compensating for the increased membrane fluidity caused by solvents (Heipieper et al., 1992; Sikkema et al., 1995; Heipieper et al., 2003). Cis-trans isomerization is an urgent response to solvents, which is substituted by other, long-term mechanisms after cells have adapted to the solvents (Neumann et al., 2005). Genes for cis-trans isomerases were identified on genomes of various pseudomonads also of solvent-sensitive strains. The cis-trans isomerase of solvent-sensitive P. putida KT2440 showed 99.5% and 95.6% amino acid sequence identity to the corresponding enzymes of solvent-tolerant P. putida DOT-TIE and P. putida P8, respectively (Junker and Ramos, 1999). In addition, cis to trans isomerization activity was also reported in P. putida MW1200 and P. putida GP01, which are not solvent-tolerant (Chen et al., 1995b; Pinkart et al., 1996), and changes of membrane lipid composition not only were observed after solvent exposure, but also occurred as a response to many environmental and physico-chemical factors like temperature, chemicals, ions, pressure, nutrients, and growth phase (Heipieper et al., 1996; Ramos et al., 2001; Denich et al., 2003). Obviously, solvent tolerance is only one of a variety of physiological functions of cis-trans isomerases.

Solvent efflux pumps belonging to the resistance nodulation division family (Murakami and Yamaguchi, 2003) extrude organic solvent molecules from the inner membrane into the extracellular space (Kieboom et al., 1998a; Kieboom et al., 1998b; Ramos et al., 1998; Rojas et al., 2001). Such translocases are proposed to span both the inner and the outer membranes (Koronakis et al., 2000; Murakami et al., 2002) and act in an energy-dependent way (coupled to the proton motive force) as a long-term response to incubation with organic solvents. For adapted cells of P. putida S12 (preincubated with sublethal concentrations of solvents), the measured influx of (radioactively labeled) toluene was two-fold lower than for nonadapted cells or in the presence of solvent pump inhibitors leading to energy uncoupling (Isken and de Bont, 1996). In nonadapted as compared to adapted P. putida S12 and P. putida DOT-T1E, toluene accumulated to two- and five-fold higher amounts, respectively, presumably mainly in
the cell membranes (Isken and de Bont, 1996; Ramos et al., 1997). It can be speculated that solvent efflux pumps, in addition to their solvent pumping activity, contribute to solvent tolerance by stabilizing the membrane structure (analogous to the structural role of porin proteins (Woodruff and Hancock, 1989)). This combined effect would explain the hypersensitivity of the efflux pump knockout mutants to toluene and emphasize the important role of efflux pumps in long-term solvent tolerance (Rojas et al., 2001).

As it can be inferred from the inducibility of efflux pump activity, adaptation seems to be an important prerequisite for a strain to develop a solvent tolerance phenotype. Solvent-tolerant *P. putida* strains cultivated in the presence of sublethal concentrations of organic solvents (Weber et al., 1993; Heipieper and de Bont, 1994; Ramos et al., 1997; Ramos et al., 1998; Neumann et al., 2005; Neumann et al., 2006) or on acetic acid (Weber et al., 1993) show high viabilities and relatively short growth lag phases upon incubation with a second liquid phase of toxic solvents, whereas only a fraction of cells survives such a shock, by e.g. toluene, without preexposure (Junker and Ramos, 1999; Duque et al., 2001). Yet, the genes of *cis-trans* isomerase and of the most important solvent efflux pump were shown to be expressed constitutively in *P. putida* DOT-T1E (Junker and Ramos, 1999; Rojas et al., 2003; Guazzaroni et al., 2004; Guazzaroni et al., 2005). The apparent need for adaptation in order to achieve effective solvent tolerance suggests a regulation by (still unknown) global factors that are activated upon incubation of the cells with organic solvents (Pedrotta and Witholt, 1999; Duque et al., 2001; Ramos et al., 2002).

In general, it is difficult to estimate the actual contributions of *cis-trans* isomerase, solvent efflux pumps, and other possible mechanisms to solvent tolerance, which ultimately enables the bacteria to grow in the presence of toxic organic solvents.

1.6.2.2 Suitability of solvent-tolerant strains for industrial applications

What are the implications of the described solvent tolerance phenotype for the use of solvent-tolerant bacteria in two-liquid-phase biotransformations? *P. putida* MC2 was used for the production of 3-methylcatechol from glucose in the presence of a second phase of octanol (Hüsken et al., 2001b). The process showed a long production lag phase of 40 hours and about 20 hours when mineral medium and LB complex medium were used, respectively. Although increasing the concentrations of the LB medium components did not improve 3-methylcatechol production, an increased energy demand for *de novo* syntheses of complex
metabolic precursors could explain the extension of the production lag phase in mineral medium. However, the use of complex media can complicate downstream processing due to the presence of high concentrations of undefined medium components. Mineral media generally contain only a small number of defined components and, in contrast to complex media, allow cultivation to high cell densities, which is a prerequisite to obtain high volumetric biotransformation rates (Riesenberg, 1991).

The presence of toluene decreased the biomass yield and increased maintenance energy demands of \textit{P. putida} S12 (Isken et al., 1999). Proteome analysis of \textit{P. putida} DOT-T1E and \textit{P. putida} S12 revealed an upregulation of proteins involved in energy metabolism as a consequence of incubation with toluene (Segura et al., 2005; Volkers et al., 2006). \textit{P. putida} DOT-T1E cells are able to grow in the presence of a second liquid phase of decanol or nonanol without significant growth inhibition after preexposure to sublethal concentrations of organic solvents (Rojas et al., 2004; Neumann et al., 2005). However, growth is completely inhibited in the presence of a second octanol phase, although adapted cells survived for at least 24 hours. Octanol accumulates to higher maximum membrane concentrations (588 mM) as compared to decanol (379 mM) (Neumann et al., 2005). Apparently, energy generation of \textit{P. putida} DOT-T1E was sufficient to reduce decanol, nonanol, or octanol concentrations in the membranes below lethal levels. The increased energy demand for the extrusion of octanol, as compared to decanol and nonanol extrusion, however, did not allow cell growth. An engineered strain of \textit{P. putida} S12 used for the production of phenol from glucose was able to grow in the presence of a second phase of octanol during fed-batch cultivation under nitrogen limited conditions, suggesting a higher level of energy metabolism of \textit{P. putida} S12 as compared to \textit{P. putida} DOT-T1E (Wierckx et al., 2005).

Although stable growth in the presence of a second phase of octanol or toluene can be achieved by the refinement of adaptation and cultivation procedures, these observations indicate that the high energy demand for solvent tolerance may limit or prevent (energy dependent) biocatalysis. Indeed, production rates of two-liquid-phase biotransformation processes with octanol as a second phase are rather low. For example, volumetric product formation rates (based on the totals of product concentration, working volume, and biotransformation time) for 3-methylcatechol synthesis are 0.054 and 0.012 g liter$^{-1}$ h$^{-1}$ with toluene (Hüsken et al., 2001b) and \textit{m}-xylene (Rojas et al., 2004) as the starting compounds, respectively, and 0.008 g liter$^{-1}$ h$^{-1}$ for the synthesis of phenol from glucose (Wierckx et al., 2005). Here, the implementation of membranes to decrease phase toxicity or mathematical models may be useful to improve such processes (Hüsken et al., 2002; Hüsken et al., 2003).
Heterologous expression of genes encoding solvent tolerance mechanisms in host strains that provide an increased metabolic activity might be a promising approach to generate competitive solvent-tolerant biocatalysts. Broad host range plasmids have been constructed encoding genes of cis-trans isomerase from solvent-tolerant *P. putida* P8 (Holtwick *et al.*, 1997) or *P. putida* DOT-TIE (Junker and Ramos, 1999) or solvent efflux pump genes from *P. putida* S12 (Kieboom *et al.*, 1998a). Thereby, cells of a solvent-sensitive *P. putida* strain overproducing a recombinant solvent efflux pump were able to tolerate higher toluene concentrations than cells of the parental strain (Kieboom *et al.*, 1998a). A cis-trans isomerase knockout mutant of *P. putida* DOT-TIE incubated with sublethal concentrations of toluene provided a longer growth lag phase than the complemented mutant overproducing recombinant cis-trans isomerase (Junker and Ramos, 1999). Such constructs may as well be applicable to bacterial strains different from *Pseudomonas*. This was shown by the overproduction of cis-trans isomerase in *E. coli*, which enabled the *E. coli* strain to synthesize trans-unsaturated fatty acids (Holtwick *et al.*, 1997). The high level of energy metabolism of α-proteobacteria, such as *Paracoccus versutus* (see Section 1.4), might enable both stable solvent tolerance and the supply of sufficient reducing equivalents for oxidative biocatalysis.

To be feasible for industrial implementation, bioprocesses require reproducible cell growth and high productivities. Unproductive time periods due to adaptation and cell growth inhibition still make biotransformations based on solvent-tolerant *P. putida* strains and octanol or toluene as second phases rather unattractive. Decanol was proposed to be a useful second liquid phase in biotransformations with *P. putida* DOT-TIE since it significantly increased the overall volumetric 3-methylcatechol accumulation rate (0.134 g liter⁻¹ h⁻¹) as compared to the rate achieved with octanol as a second phase (0.012 g liter⁻¹ h⁻¹) and allowed cell growth (Rojas *et al.*, 2004; Neumann *et al.*, 2005). However, also *E. coli* W3110 grows in mineral medium containing a second phase of heptane, which has the same logP<sub>ow</sub> as decanol (logP<sub>ow</sub>, 4.0; (Favre-Bulle *et al.*, 1991)). In addition, the saturation ratio of fatty acids in *E. coli* membranes did not change significantly in the presence of decanol or nonanol, which underlines that such organic solvents also can be tolerated by solvent-sensitive microorganisms such as *E. coli* (Sullivan *et al.*, 1979).

Overall, no clear benefit of the use of solvent-tolerant *P. putida* for redox biocatalysis has been found to date. Apparently, the solvent tolerance potential of *P. putida* strains can not yet be fully exploited for economical two-liquid-phase bioprocesses based on organic solvents that are not tolerated by "normal" bacteria.
1.7 Conclusions and future prospects

Metabolically active cells are a prerequisite for efficient redox biocatalysis, since they enable the synthesis of high concentrations of functional enzymes, cofactor regeneration, and extrusion of toxic chemicals by solvent efflux pumps in solvent-tolerant bacteria. Strains providing a higher NAD(P)H regeneration capacity than *E. coli* are interesting candidates as hosts for NAD(P)H-dependent biotransformations. However, high respiration activity may limit the availability of oxygen and NAD(P)H for oxygenase-based biocatalysis. Oxygen transfer might be increased by improving the reactor design or by reaction engineering, e.g., via the addition of hydrogen peroxide and catalase to the cell broth.

Enzymatic background activity of metabolically active cells can catalyze the degradation of substrates and products as well as side-product formation. The use of bacterial strains that provide a defined metabolic background compatible with the desired biotransformation is, therefore, favorable but might require preliminary screening and characterization of new or metabolic engineering of known strains. More sophisticated tools for heterologous gene expression in strains different from *E. coli* are required in order to use recombinants based on exotic bacterial hosts as biocatalysts in a routine way.

A second phase of an organic solvent in two-liquid-phase biotransformation allows the production of high amounts of toxic products, stabilizes reactants, and increases oxygen transfer. However, the selection of a suitable organic solvent is not trivial and depends on parameters like biocompatibility, extraction properties, and practical handling. The use of solvent-tolerant bacteria in two-liquid-phase biotransformations with organic solvents of intermediate polarity, such as toluene, may allow the synthesis of a broader range of products. However, critical factors, such as productivity lag phases, growth inhibition, and enduring adaptation procedures, have to be carefully evaluated in order to assess the suitability of solvent-tolerant bacteria for industrial applications.

This chapter elucidates the importance to improve and intensify a process by engineering efforts on the molecular, physiological, and reaction levels. Advances in the field of microbiology will provide an increasing number of enzymes and microorganisms potentially useful for redox biocatalysis. The use of host bacteria optimized for both the catalysis of the desired reaction and the applicability in an appropriate process setup will further improve the productivity of redox biocatalysis.
1.8 Scope of the thesis

In spite of the enormous diversity of bacterial species only a small selection, mainly *Escherichia coli* strains, are used as hosts in recombinant biocatalysis. The catabolic potential and the organic solvent tolerance make *Pseudomonas putida* strains interesting candidates as hosts. This work aimed to investigate effects of microbial physiology on the catalytic efficiency of biotransformations. A better understanding of interactions between the host metabolism, enzyme synthesis, and reaction catalysis might be used for the optimization of heterologous gene expression, to increase the stability of substrates, products, and the biocatalyst, to facilitate cofactor regeneration, and to increase catalytic efficiency. If successful, this work could serve to select or design bacterial host strains that are better suited for the desired biocatalytic application resulting in higher process stability and efficiency.

Chapter 2 compares the catabolic activities of *E. coli* and *P. putida* strains towards nitroaromatics and their impacts on the efficiency of the xylene monooxygenase catalyzed multistep oxidation of *m*-nitrotoluene to *m*-nitrobenzyl alcohol, *m*-nitrobenzaldehyde, and *m*-nitrobenzoic acid. In Chapter 3, the construction of plasmids for the synthesis of monooxygenases in a broad range of hosts is described. The constructs were used to systematically investigate the reproducibility of enzyme synthesis and activity in recombinant *E. coli* and *P. putida* strains used as biocatalysts for the selective oxidation of aromatic and heteroaromatic compounds. Chapter 4 investigates the biocompatibility and the physiological response of *E. coli* JM101 to 1-decanol in order to evaluate the need for solvent-tolerant *P. putida* strains used as hosts in two-liquid-phase biotransformations with 1-decanol as second phase. Chapter 5 investigates the feasibility of the *E. coli* central metabolism to be coupled with recombinant xylene monooxygenase and pyruvate decarboxylase for the production of *(R)-phenylacetylcarnbinol* from toluene. Metabolic and process engineering aspects were taken into consideration. Finally, Chapter 6 concludes the knowledge gained in this work with respect to the impacts for the design of future biocatalytic applications and for the development towards the “ideal” host strain.
Chapter 2: Suitability of recombinant *Escherichia coli* and *Pseudomonas putida* strains for selective biotransformation of *m*-nitrotoluene by xylene monooxygenase

Summary

*Escherichia coli* JM101 (pSPZ3) containing xylene monooxygenase (XMO) from *Pseudomonas putida* mt-2, catalyzes specific oxidations and reductions of *m*-nitrotoluene and derivatives thereof. In addition to reactions catalyzed by XMO, we focused on biotransformations by native enzymes of the *E. coli* host and their effect on overall biocatalyst performance. While *m*-nitrotoluene was consecutively oxygenated to *m*-nitrobenzyl alcohol, *m*-nitrobenzaldehyde, and *m*-nitrobenzoic acid by XMO, the oxidation was counteracted by alcohol dehydrogenase(s) from the *E. coli* host, which reduced *m*-nitrobenzaldehyde to *m*-nitrobenzyl alcohol. Furthermore, the enzymatic background of the host reduced the nitro groups of the reactants resulting in the formation of aromatic amines, which were shown to effectively inhibit XMO in a reversible fashion. Host-intrinsic oxidoreductases and their reaction products had a major effect on the activity of XMO during biocatalysis of *m*-nitrotoluene. *P. putida* DOT-TIE and *P. putida* PpS81 were compared to *E. coli* JM101 as alternative hosts for XMO. These promising strains contained an additional dehydrogenase that oxidized *m*-nitrobenzaldehyde to the corresponding acid but catalyzed the formation of XMO-inhibiting aromatic amines at a significantly lower level than *E. coli* JM101.

Introduction

Microbial enzymes are useful catalysts for the degradation of organic pollutants in bioremediation but also for the synthesis of added-value products in biocatalytic applications. Various large-scale processes based on bacterial enzymes have been established (Kiener, 1992; Glöckler and Roduit, 1996; Liese et al., 2000; Straathof et al., 2002), which illustrate the increasing impact of biocatalysis in the chemical industry (Schmid et al., 2001a). Heterologous gene expression has developed to a standard technology for increasing and controlling enzyme activities in bioprocesses. It allows catalysis of a variety of reactions in a limited number of bacterial strains used as recombinant hosts. The focus on a few suitable hosts is emphasized, e.g., by the Plug-Bug concept (DSM, Heerlen, The Netherlands). Of these strains, *Escherichia coli* strains are easily accessible for genetic and biochemical engineering and provide high metabolic activity for cofactor regeneration. Their use as recombinant hosts is especially favorable in cofactor-dependent oxygenase-based processes (Bühler and Schmid, 2004) for the conversion of various aromatic hydrocarbons to industrially relevant products (Wubbolts et al., 1994a; Panke et al., 2002; Bühler et al.,
Host selection for selective biotransformation of m-nitrotoluene

2003b). E. coli JM101 (pSPZ3) (Panke et al., 1999b) contains xylene monooxygenase (XMO) from P. putida mt-2 and oxidizes the methyl groups of a variety of toluene and xylene derivatives to the corresponding alcohols, aldehydes, and acids (Delgado et al., 1992; Wubbolts et al., 1994b; Bühler et al., 2000).

Nitroaromatics are widely used in large amounts as synthetic intermediates, dyes, pesticides, pharmaceuticals, and explosives (Hartter, 1985; Spain, 1995). Their electrophilic character makes them susceptible to reduction by all kinds of microbial systems (Rieger and Knackmuss, 1995). Numerous bacterial strains, such as Pseudomonas species (Schackmann and Müller, 1991; Ali-Sadat et al., 1995) and E. coli (Diaz et al., 2001), provide enzymes that are able to transform nitro groups of aromatic compounds under aerobic conditions. This indicates that biocatalysis of nitroaromatics might be complicated by influences of the enzymatic background activity of the host strain (Wilkinson et al., 1996).

In this study, we were interested in the suitability of E. coli JM101 (pSPZ3) for the oxidation of m-nitrotoluene by XMO. We investigated the enzymatic background activity of this biocatalyst and parameters determining its efficiency and specificity on a technical scale. A comparison with solvent-tolerant P. putida DOT-T1E (Ramos et al., 1995) and P. putida PpS81 (Grund et al., 1975), which is deficient for an alcohol dehydrogenase, opened new perspectives for recombinant P. putida strains as efficient biocatalysts in XMO-based processes with nitroaromatic compounds.

Materials and methods

Chemicals. Chemicals were obtained from Fluka (Buchs, Switzerland) [m-nitrotoluene, ≥99% pure; m-nitrobenzyl alcohol, ≥95%; m-nitrobenzaldehyde, ≥97%; m-nitrobenzoic acid, >98%; m-toluidine, >99.0%; m-aminobenzyl alcohol, ≥97%; m-aminobenzoic acid, ≥97%; bis(2-ethylhexyl)phthalate, 97%], Riedel-de Haén (Buchs, Switzerland) [p-dimethylaminobenzaldehyde, ≥99%], Aldrich (Buchs, Switzerland) [dicyclopentyl ketone, 95%], and Acros Organics (Geel, Belgium) [n-octane, ≥98.5%].

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids are listed in Table 2.1. Bacteria were grown in Luria-Bertani (LB) complex medium (Difco, Detroit, Mich.), M9* mineral medium (identical to M9 mineral medium (Sambrook et al., 1989) except that it contained three times more phosphate salts in order to increase the buffer capacity and did not contain calcium chloride) (Panke et al., 1999b), and RB mineral medium (Bühler et al., 2003a). Both mineral media were supplemented with 1 ml/liter USFec-
trace element solution (Bühler et al., 2003a). Antibiotics (kanamycin, 50 mg/liter; rifampin, 20 mg/liter) and thiamine (10 mg/liter) were added when appropriate. The pH of the M9* mineral medium was adjusted to 7.4 with 10 M NaOH before use in shaking flasks. All cultivations were performed at 30°C and glucose (0.5% (wt/vol)) was used as carbon source.

Table 2.1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td>thi Δlac-proAB F’traD36 proAB+ lacI9</td>
<td>(Messing, 1979)</td>
</tr>
<tr>
<td></td>
<td>lacZΔM15</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOT-T1E</td>
<td>Solvent tolerant, Rif’</td>
<td>(Ramos et al., 1995)</td>
</tr>
<tr>
<td>PpS81</td>
<td>alcA mutant</td>
<td>(Grund et al., 1975)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSPZ3</td>
<td>pBR322-derived, encoding XMO genes, xylM and xylA under control of the alk regulatory system, Km’</td>
<td>(Panke et al., 1999b)</td>
</tr>
</tbody>
</table>

Rif, rifampin; Km, kanamycin

**Whole-cell activity assays.** Whole-cell biotransformations (1-ml-scale) with resting cells of *E. coli* JM101 (pSPZ3) were used to determine XMO activity for the sequential oxidation of *m*-nitrotoluene by using a procedure described elsewhere (Bühler et al., 2000). Bacteria were grown in glucose-containing M9* mineral medium, and XMO synthesis was induced with 0.1% (vol/vol) *n*-octane. After being harvested, bacteria were resuspended to about 2 g cell dry weight (CDW) per liter in potassium phosphate buffer (50 mM, pH 7.4) containing glucose. 1.0-ml portions of cell suspension were incubated with 1.0 mM *m*-nitrotoluene on a rotary shaker at 250 rpm and 30°C. Product formation was monitored by stopping the reaction at different time points by acidification with 40 μl of 10% (vol/vol) perchloric acid solution. After centrifugation, the supernatant was analyzed for nitroaromatics by reversed-phase high-performance liquid chromatography (RP-HPLC) (see below). Initial activities were calculated from the amount of product formed after 5 min of biotransformation and were expressed in units per gram CDW. One unit is defined as the enzyme activity that forms 1 μmol of product per minute. The experiments were repeated independently at least once. *m*-Nitrobenzyl alcohol or *m*-nitrobenzaldehyde at 1.0 mM served as the substrate to determine initial activities of the second or third XMO-catalyzed reaction step, respectively.
For studies of inhibition of XMO activity, cell suspensions were incubated with 1.0 mM substrate together with the inhibitor at various concentrations from 0 to 20 mM.

Whole-cell activity assays were also performed using E. coli JM101, P. putida DOT-T1E, and P. putida PpS81 wild-type strains in order to determine enzyme activities converting \(m\)-nitrobenzaldehyde to \(m\)-nitrobenzyl alcohol or \(m\)-nitrobenzoic acid.

Analogously, the wild-type strains were incubated with 1.0 mM \(m\)-nitrotoluene, \(m\)-nitrobenzyl alcohol, \(m\)-nitrobenzaldehyde, or \(m\)-nitrobenzoic acid to determine the rate of formation of aromatic amines. The incubation time was prolonged to 90 min due to the low formation rates. Aromatic amines were detected by using Ehrlich’s reagent (see below).

**Two-liquid-phase biotransformation.** Biotransformations in the presence of a second, organic liquid phase were performed on a 2-liter-scale using a reactor setup and a procedure described earlier (Bühler et al., 2003a). A 100-ml overnight culture of E. coli JM101 (pSPZ3) in RB medium was used as inoculum for a bioreactor containing 900 ml RB medium with 0.7 % (wt/vol) glucose as carbon source. Cells grew in batch mode at 30°C over night. The pH was maintained constant at 7.4 by regulated addition of aqueous solutions of phosphoric acid and ammonium hydroxide. Stirrer speed and aeration rate were set to 1500 rpm and 1 liter air per minute, respectively. Fed-batch cultivation was started by activating the feed of a solution containing 730 g glucose and 19.6 g MgSO\(_4\) \(\cdot\) 7 H\(_2\)O per liter after complete consumption of the carbon source (corresponds to time zero). The initial feed rate of 5.9 g glucose/h was increased stepwise during fed-batch cultivation (Fig. 2.4B; Fig. 2.6B). XMO synthesis was induced by the addition of dicyclopropyl ketone to a concentration of 0.02% (vol/vol) in the aqueous phase and of 60 mmol \(n\)-octane in the presence of the second liquid phase. Biotransformation was started by the addition of 1 liter of the second liquid phase, which consisted of bis(2-ethylhexyl)phthalate (BEHP) and 50 mmol \(m\)-nitrotoluene as substrate. The stirrer speed and aeration rate were increased to 2000 rpm and 2 liters per minute, respectively. After separation of the aqueous and organic phase of samples by centrifugation, nitroaromatics were detected by RP-HPLC and gas chromatography (GC) (see below). Glucose and acetic acid concentrations were determined as described elsewhere (Bühler et al., 2003a). The cell concentration was determined spectrophotometrically at 450 nm with a Novaspec II spectrophotometer from Pharmacia Biotech as described elsewhere (Witholt, 1972). One absorption unit at 450 nm corresponded to a cell dry weight of 0.29 g/liter.

**Analytical procedures.** \(m\)-Nitrotoluene, \(m\)-nitrobenzyl alcohol, \(m\)-nitrobenzaldehyde, and \(m\)-nitrobenzoic acid in aqueous samples were separated with an RP-HPLC instrument from
Merck Hitachi (interface D-7000, UV-detector L-7400, pump L-7100, autosampler L-7200) equipped with a Nucleosil C18 column (pore size, 100 Å; particle size, 5 μm; inner diameter, 4 mm; length, 25 cm) from Macherey-Nagel (Oensingen, Switzerland). The mobile phase consisted of acetonitrile and H2O containing 0.1% perchloric acid. The elution profile was 35 to 43% acetonitrile over 5 min, followed by 43 to 80% over 5 min, and then 80% acetonitrile isocratic for 2 min. The flow rate was 1.0 ml/min. Detection occurred at 210 nm. In addition, aqueous samples were extracted with an equal volume of diethyl ether as described elsewhere (Bühler et al., 2000) followed by analysis of the ether phase by GC (see below) in order to determine concentrations of nitroaromatics complementary to RP-HPLC analysis.

Samples from the organic phase of the two-liquid-phase biotransformations were diluted 25-fold in ice-cold diethyl ether containing 0.1 mM n-decane as an internal standard and were dried over sodium sulfate. m-Nitrotoluene, m-nitrobenzyl alcohol, and m-nitrobenzaldehyde were separated using a GC instrument from Fisons Instruments equipped with an OPTIMA-5 fused silica capillary column (length, 25 m; inner diameter, 0.32 mm; film thickness, 0.25 μm) from Macherey-Nagel (Oensingen, Switzerland). The temperature profile was as follows: isotherm at 40°C for 2 min, from 40 to 280°C at 18°C/min, and then 280°C isotherm for 5 min. Splitless injection with hydrogen as the carrier gas was used. Detection occurred by flame ionization. Identification and quantification of the substances were done by comparison with commercially available standards.

Aromatic amines in samples from whole-cell activity assays were detected by addition of Ehrlich’s reagent, consisting of 20 g/liter p-dimethylaminobenzaldehyde in 20% hydrochloric acid (Bogensberger et al., 1998). p-Dimethylaminobenzaldehyde reacts with primary aromatic amines to form yellow imines in acidic environments (Fig. 2.1A) (Kolsek et al., 1957). Twenty microliters of Ehrlich’s reagent was added per ml cell-free supernatant of aqueous samples. Formation of yellow reaction products was monitored by eye or spectrophotometrically at 436 nm, the corresponding absorption maximum (Fig. 2.1B). m-Toluidine, m-aminobenzyl alcohol, and m-aminobenzoic acid served as standards for the expected amines produced by the host from m-nitrotoluene, m-nitrobenzyl alcohol, and m-nitrobenzoic acid, respectively. m-Aminobenzaldehyde was not available as a standard due to its instability (Feng et al., 1998). Aromatic amines formed from m-nitrobenzaldehyde were quantified using an equimolar mixture of m-toluidine, m-aminobenzyl alcohol, and m-aminobenzoic acid as a standard, which might have influenced the quantification.
Results

Biotransformation of m-nitrotoluene by *E. coli* JM101 (pSPZ3). Resting cells of *E. coli* JM101 (pSPZ3) containing XMO were incubated with 1.0 mM of *m*-nitrotoluene, which was oxidized sequentially to *m*-nitrobenzyl alcohol, *m*-nitrobenzaldehyde, and *m*-nitrobenzoic acid at maximum formation rates of 105, 60, and 6 U/g CDW, respectively (Fig. 2.2). When *m*-nitrobenzyl alcohol was used as a substrate, *m*-nitrobenzaldehyde was formed at a rate of
90 U/g CDW. With \( m \)-nitrobenzaldehyde as a substrate, \( m \)-nitrobenzoic acid was formed at 13 U/g CDW. In addition, \( m \)-nitrobenzaldehyde was reduced to the corresponding alcohol in *E. coli* JM101 (pSPZ3) (Fig. 2.2).

Metabolically active cells are a prerequisite for stable biocatalytic activity since they maintain both cofactor regeneration and enzymes synthesis. However, \( m \)-nitrotoluene and its oxidized derivatives are supposed to be toxic to bacteria. Thus, the concentration-dependent effect of nitroaromatics on growth of *E. coli* JM101 was investigated. \( m \)-Nitrobenzaldehyde was the most toxic compound tested by completely inhibiting growth at 1.25 mM, followed by \( m \)-nitrotoluene, \( m \)-nitrobenezyl alcohol, and \( m \)-nitrobenzoic acid (Fig. 2.3). Although already 2.5 mM \( m \)-nitrobenzoic acid reduced cell growth by more than 80%, complete inhibition by this compound was not observed at saturating concentrations.

![Fig. 2.2. Multistep oxygenation of \( m \)-nitrotoluene by *E. coli* JM101 (pSPZ3).](image)

The assay was performed with resting cells (1.7 g CDW/liter) as described in “Materials and methods”. \( m \)-Nitrotoluene at 1.0 mM (●) served as a substrate. \( m \)-Nitrobenezyl alcohol (■), \( m \)-nitrobenzaldehyde (▲), and \( m \)-nitrobenzoic acid (●) were formed as consecutive products. ×, sum of nitroaromatic reactant concentrations.
Fig. 2.3. Growth of *E. coli* JM101 incubated with different concentrations of nitroaromatics. A culture of *E. coli* JM101 cells exponentially growing in RB medium (0.5% (wt/vol) glucose; pH 7.4) at 37°C was split into subcultures, which were incubated with different concentrations of *m*-nitrotoluene, *m*-nitrobenzyl alcohol, *m*-nitrobenzaldehyde, or *m*-nitrobenzoic acid, followed by the determination of growth rates. The dashed lines represent supersaturating concentrations of *m*-nitrotoluene and *m*-nitrobenzyl alcohol at 30°C and 20°C, respectively (cf. Table 2.2).

The use of a two-liquid-phase biotransformation system, involving e.g. BEHP as a second, organic phase, can reduce growth inhibiting effects of toxic substrate/products by extracting them from the aqueous, cell-containing phase. The partition coefficients for *m*-nitrotoluene, *m*-nitrobenzyl alcohol, *m*-nitrobenzaldehyde, and *m*-nitrobenzoic acid in a BEHP–medium mixture allowed the estimation of the maximal substrate/product concentrations that are supported by the *E. coli* JM101 cells during such a two-liquid-phase biotransformation. *m*-Nitrobenzyl alcohol and *m*-nitrobenzaldehyde are lethal for the bacteria at significantly lower overall concentrations as compared to *m*-nitrotoluene in such a two-phase system (Table 2.2).
Table 2.2. Aqueous solubility, toxicity, and partitioning of nitroaromatics in a two-liquid-phase system with BEHP as the second phase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximum aqueous solubility(^a) [mM]</th>
<th>Critical aqueous concentration(^b) [mM]</th>
<th>Partition coefficient (BEHP–RB)(^c)</th>
<th>Critical concentration in emulsion(^d) [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(m)-nitrotoluene</td>
<td>3.9</td>
<td>2.5 – 5.0</td>
<td>~1500</td>
<td>3800 – 7500</td>
</tr>
<tr>
<td>(m)-nitrobenzyl alcohol</td>
<td>3.8</td>
<td>7.5 – 10.0</td>
<td>9.8 ± 0.4</td>
<td>79 – 112</td>
</tr>
<tr>
<td>(m)-nitrobenzaldehyde</td>
<td>10.8</td>
<td>0.625 – 1.25</td>
<td>73.0 ± 3.2</td>
<td>44 – 97</td>
</tr>
<tr>
<td>(m)-nitrobenzoic acid</td>
<td>20.4</td>
<td>n.d.(^e)</td>
<td>0.90 ± 0.06</td>
<td>n.d.(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Solubilities of \(m\)-nitrotoluene (at 30°C), \(m\)-nitrobenzyl alcohol (at 20°C), \(m\)-nitrobenzaldehyde (at 25°C), and \(m\)-nitrobenzoic acid (at 24.9°C) were obtained from (Benes and Dohnal, 1999), (Carter et al., 1958), (Goldschmidt and Sunde, 1906), and (Biswas and Lahiri, 1994), respectively.

\(^b\) The minimal concentrations at which growth of \(E.\ coli\) JM101 is completely inhibited, estimated from Fig. 2.3.

\(^c\) Partition coefficients were determined as described earlier (Bühler et al., 2003a). The aqueous phase consisted of RB medium (0.5% (wt/vol) glucose, pH 7.4).

\(^d\) Estimated minimal overall concentrations at which growth of \(E.\ coli\) JM101 is completely inhibited in a two-liquid-phase system with BEHP as a second phase. Phase ratio, 0.5.

\(^e\) n.d., not determined, since growth of \(E.\ coli\) JM101 was not completely inhibited by saturating \(m\)-nitrobenzoic acid concentrations.

Although cell growth was not completely inhibited at saturating \(m\)-nitrobenzoic acid concentrations, an overall acid concentration of 4.6 mM results in a reduction of cell growth by more than 80% in a two-liquid-phase system due to the effective growth inhibition at soluble concentrations and the preferable partitioning to the aqueous phase.

The oxidation of \(m\)-nitrotoluene by XMO was investigated with higher substrate concentrations and over an extended time period by using growing \(E.\ coli\) JM101 (pSPZ3) in a two-liquid-phase biotransformation setup on a 2-liter-scale. XMO synthesis was started by induction 50 min before substrate addition. The course of biotransformation is shown in Fig. 2.4A. After substrate addition, cells oxidized \(m\)-nitrotoluene to the corresponding alcohol and aldehyde at rates of 40 and 23 U/g CDW, respectively. No significant oxidation to the acid was detected (<0.5 mmol). The aldehyde accumulated to a maximum of 17 mmol about four hours after feed initiation. Afterwards, it disappeared at a rate of 21 U/g CDW, whereas at the same time the corresponding alcohol was formed at a rate of 26 U/g CDW. The relatively small difference between the rates for aldehyde disappearance and alcohol formation indicated the predominant formation of \(m\)-nitrobenzyl alcohol from the aldehyde independently from XMO activity by host intrinsic enzymes of \(E.\ coli\) JM101.
Fig. 2.4. Biotransformation of m-nitrotoluene by E. coli JM101 (pSPZ3) in a two-liquid-phase system on a 2-liter scale (working volume). (A) XMO synthesis was induced 50 min before substrate addition. Biotransformation was started by the addition of the second organic phase (phase ratio, 0.5) containing 50 mmol m-nitrotoluene. After most of the substrate was converted, another 50 mmol m-nitrotoluene was added. Nitroaromatics are represented as the sum of the respective amounts in the organic and aqueous phase. The mass balance of nitroaromatics describes the overall sum of m-nitrotoluene, m-nitrobenzyl alcohol, m-nitrobenzaldehyde, and m-nitrobenzoic acid given as the percentage (moles/moles) of m-nitrotoluene added as substrate. (B) Cultivation parameters of the biotransformation. The glucose feed was increased stepwise starting at 5.9 g/h. Experimental details are described in “Materials and methods”.
The lack of \( m \)-nitrobenzyl alcohol formation in the absence of \( m \)-nitrobenzaldehyde confirmed the minor XMO activity towards the end of the biotransformation. Acetic acid accumulated to concentrations, which according to (Konstantinov et al., 1990) do not affect cell growth in a significant way (Fig. 2.4B). The process parameters are summarized in Table 2.3.

Table 2.3. Process parameters of the two-liquid-phase biotransformation of \( m \)-nitrotoluene by \( E. coli \) JM101 (pSPZ3)\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotransformation time(^b)</td>
<td>h</td>
<td>5.5</td>
</tr>
<tr>
<td>Final cell concn</td>
<td>g CDW/liter(_{aq})</td>
<td>23.1</td>
</tr>
<tr>
<td>Total ( m )-nitrotoluene amt added</td>
<td>mmol</td>
<td>99.8</td>
</tr>
<tr>
<td>Final ( m )-nitrotoluene amt</td>
<td>mmol</td>
<td>41.7</td>
</tr>
<tr>
<td>Final ( m )-nitrobenzyl alcohol amt</td>
<td>mmol</td>
<td>38.5</td>
</tr>
<tr>
<td>Final ( m )-nitrobenzaldehyde amt</td>
<td>mmol</td>
<td>0.4</td>
</tr>
<tr>
<td>Final ( m )-nitrobenzoic acid amt</td>
<td>mmol</td>
<td>0.0</td>
</tr>
<tr>
<td>Molar yield</td>
<td>%</td>
<td>48</td>
</tr>
<tr>
<td>Maximal specific XMO activity(^c)</td>
<td>U/g CDW</td>
<td>40</td>
</tr>
<tr>
<td>Maximal specific accumulation(_{alc})</td>
<td>U/g CDW</td>
<td>26</td>
</tr>
<tr>
<td>Maximal volumetric activity(_{alc})</td>
<td>U/liter(_{aq})</td>
<td>344</td>
</tr>
<tr>
<td>Average volumetric activity(_{alc})</td>
<td>U/liter(_{aq})</td>
<td>117</td>
</tr>
</tbody>
</table>
| Average productivity\(_{alc}\)                 | g/(liter\(_{tot}\) \(
\cdot\hbar\)) | 0.54  |

\(^a\) Two-liter scale (working volume); phase ratio of 0.5. \(_{aq}\), aqueous phase; \(_{tot}\), organic and aqueous phase; \(_{alc}\), referring to \( m \)-nitrobenzyl alcohol accumulation.

\(^b\) Organic phase addition is defined as the start point.

\(^c\) Based on the XMO-catalyzed formation of \( m \)-nitrobenzyl alcohol, aldehyde, and acid.

Stability of \( m \)-nitrobenzaldehyde in different bacterial strains. Resting \( E. coli \) JM101 bacteria were incubated with \( m \)-nitrobenzaldehyde in whole-cell activity assays in order to investigate the stability of this product towards host intrinsic enzyme activities. \( m \)-Nitrobenzaldehyde was reduced to \( m \)-nitrobenzyl alcohol at an initial rate of 24 U/g CDW (Fig. 2.5A).

In addition, the enzymatic background activities of other promising host strains were determined. We incubated \( m \)-nitrobenzaldehyde with solvent-tolerant \( P. putida \) DOT-TIE (Ramos et al., 1995) and \( P. putida \) PpS81, a strain which is deficient for an alcohol dehydrogenase and thus unable to oxidize alkanols (Grund et al., 1975; Bosetti et al., 1992; Mathys et al., 1999). Reductive activities for \( m \)-nitrobenzaldehyde were present in both \( Pseudomonas \) strains and formed \( m \)-nitrobenzyl alcohol at rates of 12 U/g CDW (Fig. 2.5B and C). Oxidative activities formed \( m \)-nitrobenzoic acid from \( m \)-nitrobenzaldehyde at initial
rates of 11 and 17 U/g CDW in *P. putida* DOT-TIE and *P. putida* PpS81, respectively. In *E. coli* JM101, *m*-nitrobenzaldehyde was oxidized to *m*-nitrobenzoic acid at a negligible rate below 1 U/g CDW (Fig. 2.5A). All strains under investigation showed oxidoreductase activities for the carbonyl group of *m*-nitrobenzaldehyde (Fig. 2.5D) but no oxidation of the methyl group of *m*-nitrotoluene was detected.

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

**Fig. 2.5. Stability of m-nitrobenzaldehyde in different bacterial strains.** (A) *E. coli* JM101 (1.9 g CDW/liter), (B) *P. putida* DOT-TIE (2.3 g CDW/liter), and (C) *P. putida* PpS81 (1.7 g CDW/liter) were incubated with 1.0 mM *m*-nitrobenzaldehyde (▲). *m*-Nitrobenzyl alcohol (■) and *m*-nitrobenzoic acid (●) were formed as products. ×, sum of nitroaromatic reactant concentrations. (D) Scheme of *m*-nitrobenzaldehyde-degrading reactions catalyzed by host-intrinsic enzymes of *E. coli* JM101, *P. putida* DOT-TIE, and *P. putida* PpS81.
Inhibition of xylene monooxygenase by aromatic amines, side products formed from nitroaromatics in the *E. coli* host. The two-liquid-phase biotransformation was repeated using the identical biocatalyst and protocol except for the time point of induction in order to verify whether the relatively fast decrease of XMO activity during biotransformation correlates with the presence of nitroaromatics. Instead of substrate addition 50 min after induction, cells were incubated with nitroaromatics for 3.5 h before induction of XMO synthesis. In addition to 50 mmol *m*-nitrotoluene as a substrate, the second liquid phase also contained 10 mmol each of *m*-nitrobenzyl alcohol and *m*-nitrobenzaldehyde.

The enzymatic background activity of *E. coli* JM101 (pSPZ3) reduced all *m*-nitrobenzaldehyde to the corresponding alcohol at an initial rate of 29 U/g CDW, immediately after the nitroaromatics were added (Fig. 2.6A). No formation of *m*-nitrobenzyl alcohol, *m*-nitrobenzaldehyde, or *m*-nitrobenzoic acid was detected after the delayed induction, which indicated a complete absence of XMO activity, while biomass, glucose, and acetic acid concentrations (Fig. 2.6B) were similar to those seen in the previously performed biotransformation (Fig. 2.4B). During the experiment, bacteria were withdrawn from the bioreactor and diluted 80-fold in phosphate buffer for whole-cell activity assays with *m*-nitrotoluene as a substrate. No *m*-nitrobenzyl alcohol formation was detected in samples withdrawn before induction at 4.5 h. In samples withdrawn after induction at 6.5 and 8.5 h, the respective *m*-nitrobenzyl alcohol formation rates were recovered to 75% and 49%, compared to rates obtained in whole-cell activity assays using bacteria from the same inoculum. This pointed to a reversible inhibition of XMO.

The sum of nitroaromatics steadily decreased in concentration throughout both two-liquid-phase biotransformations, independent of the time point of induction (Fig. 2.4A and Fig. 2.6A). The volatility of *m*-nitrotoluene, *m*-nitrobenzyl alcohol, and *m*-nitrobenzaldehyde was tested in a bioreactor experiment under conditions identical to the two-liquid-phase biotransformations but in the absence of bacteria. None of the tested nitroaromatics was found to be stripped out (Fig. 2.7). Thus, the observed decrease of the sum of nitroaromatics during biotransformation indicated the formation of side products from nitroaromatics by native enzymes of the *E. coli* host.
Fig. 2.6. Biotransformation by *E. coli* JM101 (pSPZ3) with induction of XMO synthesis 3.5 h after addition of nitroaromatics. (A) Biotransformation was performed using the same initial conditions as in the experiment shown in Fig. 2.4, except for the addition of *m*-nitrobenzyl alcohol and *m*-nitrobenzaldehyde with *m*-nitrotoluene in the organic phase and the delayed induction of XMO synthesis. During biotransformation, no *m*-nitrobenzoic acid was detected, and its concentration therefore is not indicated in the figure. (B) Cultivation parameters of the biotransformation.
Many microorganisms are able to reduce nitro groups to amine groups (Nishino et al., 2000; Rosser et al., 2001; Zhao and Ward, 2001; Johnson and Spain, 2003; Ye et al., 2004). In order to investigate whether \textit{E. coli} JM101 forms aromatic amines, resting cells were incubated with \textit{m}-nitrotoluene, \textit{m}-nitrobenzyl alcohol, \textit{m}-nitrobenzaldehyde, or \textit{m}-nitrobenzoic acid. Primary aromatic amines were detected as condensation products of Ehrlich’s reagent for all nitroaromatics tested in \textit{E. coli} JM101 and, with lower formation rates, also in \textit{P. putida} DOT-T1E and \textit{P. putida} PpS81 (Table 2.4). The formation of \textit{m}-aminobenzaldehyde from \textit{m}-nitrobenzaldehyde remained unclear, since a prior reduction or oxidation to \textit{m}-nitrobenzyl alcohol or \textit{m}-nitrobenzoic acid, followed by the conversion to \textit{m}-aminobenzyl alcohol or \textit{m}-aminobenzoic acid, respectively, in the tested bacteria might have occurred.

![Graph](image-url)

**Fig. 2.7. Volatility of nitroaromatics in the two-liquid-phase setup.** \textit{m}-Nitrotoluene, \textit{m}-nitrobenzyl alcohol, and \textit{m}-nitrobenzaldehyde were incubated in a two-liquid-phase system on a 2-liter scale under identical conditions as in the biotransformation experiments (c.f. Fig. 2.4 and Fig. 2.6) (aeration rate, 2 liters per min; stirrer rate, 2000 rpm; 30°C; pH 7.4; phase ratio, 0.5) except for the absence of bacteria and glucose.
Table 2.4. Formation of aromatic amines from the corresponding nitroaromatics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aromatic amine formation rate (U/g CDW) on a</th>
<th>m-Nitrotoluene</th>
<th>m-Nitrobenzyl alcohol</th>
<th>m-Nitrobenzaldehyde</th>
<th>m-Nitrobenzoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM101</td>
<td>1.18 ± 0.04</td>
<td>0.70 ± 0.11</td>
<td>2.63 ± 0.24</td>
<td>3.39 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>P. putida DOT-T1E</td>
<td>0.14 ± 0.03</td>
<td>0.10 ± 0.03</td>
<td>0.55 ± 0.06</td>
<td>0.55 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>P. putida PpS81</td>
<td>0.09 ± 0.07</td>
<td>0.02 ± 0.02</td>
<td>0.15 ± 0.06</td>
<td>0.09 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

a Formation rates (means ± standard deviations) are based on aromatic amine concentrations detected by Ehrlich’s reagent in whole-cell activity assays using resting cells (~2 g CDW/liter). See “Materials and methods” for details. One unit is the enzyme activity that forms 1 μmol of product per min.

We investigated a possible relationship between the inhibition of XMO observed during the two-liquid-phase biotransformation and the formation of aromatic amines. Therefore, whole-cell activity assays were performed using E. coli JM101 (pSPZ3) as a biocatalyst with m-nitrotoluene as the substrate in the presence of various concentrations of the expected side products m-toluidine, m-aminobenzyl alcohol, and m-aminobenzoic acid (Fig. 2.8).

![Fig. 2.8. Effect of aromatic amines on the XMO-catalyzed m-nitrotoluene oxidation to the corresponding alcohol.](image-url)

The remaining activities of the first XMO-catalyzed reaction step in the presence of different concentrations of m-toluidine (●), m-aminobenzyl alcohol (■), and m-aminobenzoic acid (▲) in resting E. coli JM101 (pSPZ3) (0.8 to 0.9 g CDW/liter) are represented relative to the noninhibited XMO activity.
A lowered cell density (0.8 to 0.9 g CDW/liter) used in this experiment allowed focusing on the first XMO-catalyzed reaction step. All three aromatic amines inhibited the XMO-catalyzed oxidation of \textit{m}-nitrotoluene (Fig. 2.8). \textit{m}-Toluidine was found to be the most effective inhibitor, completely repressing \textit{m}-nitrobenzyl alcohol formation at concentrations above 2.0 mM.

Incubation with \textit{m}-nitrotoluene, \textit{m}-nitrobenzyl alcohol, or \textit{m}-nitrobenzaldehyde as substrate in combination with 200 \textmu M \textit{m}-toluidine, 8 mM \textit{m}-aminobenzyl alcohol, or 20 mM \textit{m}-aminobenzoic acid resulted in inhibition of each of the XMO-catalyzed reaction steps in \textit{E. coli} JM101 (pSPZ3) (Table 2.5). The applied \textit{m}-toluidine and \textit{m}-aminobenzyl alcohol concentrations completely inhibited the formation of \textit{m}-nitrobenzaldehyde from the corresponding alcohol. The determined residual XMO activities, however, might have been influenced by host-intrinsic enzymes of the \textit{E. coli} host which reduced \textit{m}-nitrobenzaldehyde to \textit{m}-nitrobenzyl alcohol (Fig. 2.5A).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Residual activity (%) for$^{a}$:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{m}-Nitrotoluene</td>
</tr>
<tr>
<td>\textit{m}-Toluidine, 200 \textmu M</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>\textit{m}-Aminobenzyl alcohol, 8 mM</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>\textit{m}-Aminobenzoic acid, 20 mM</td>
<td>83 ± 14</td>
</tr>
</tbody>
</table>

$^{a}$ Residual activities (means ± standard deviations) of \textit{E. coli} JM101 (pSPZ3) as a biocatalyst (1.7 g CDW/liter) are given as percentage of the activities in the absence of aromatic amines.

Discussion

The increasing knowledge about microbial metabolic pathways, the involved enzymes, and influences of environmental conditions on biodegradation (Ellis \textit{et al.}, 1999) might lead to the characterization of preferred groups of microorganisms as hosts for the biocatalysis of specific reaction types. This development might simplify the selection and design of bacterial host strains for future applications (Kieslich, 1993) and motivated our investigation of the biotransformation of \textit{m}-nitrotoluene by recombinant \textit{E. coli} containing XMO.

The successive oxidation of \textit{m}-nitrotoluene to the corresponding alcohol, aldehyde, and acid (Fig. 2.2) revealed an XMO-catalyzed multistep oxygenation, as reported for toluene and
pseudocumene (Bühler et al., 2000). The results of the two-liquid-phase biotransformation of m-nitrotoluene by E. coli JM101 (pSPZ3) were promising and illustrate the usefulness of this in situ extraction system for the accumulation of m-nitrobenzyl alcohol in technically relevant concentrations (Table 2.3). The further oxidation to m-nitrobenzaldehyde and m-nitrobenzoic acid, however, was hindered by the activity of host-intrinsic enzymes, which turned out to play a key role in reaction selectivity (Fig. 2.9).

**Effects of aromatic amine formation on xylene monooxygenase activity.** Degradation of nitroaromatics can be initialized by the enzymatic reduction of the nitro to the amine group via nitroso and hydroxylamino groups in various microorganisms (Schackmann and Müller, 1991; Marvin-Sikkema and de Bont, 1994; Cerniglia and Somerville, 1995; Caballero et al., 2005). E. coli also contains a set of oxygen-insensitive nitroreductases (Bryant et al., 1981; Whiteway et al., 1998), which aerobically reduce nitro aromatic compounds to the corresponding amines (Diaz et al., 2001). Although the catalyzed reduction of aromatic hydroxylamines to the corresponding amines in E. coli is still unclear (Nishino et al., 2000; Kadiyala et al., 2003), we observed aromatic amine formation from all nitroaromatics tested (Table 2.4). However, it remains unclear whether the assay based on Ehrlich’s reagent can distinguish between aromatic amines and hydroxylamines. The simple detection of aromatic amines as yellow condensation products of Ehrlich’s reagent makes this method a useful tool for future investigations on the biodegradation of nitroaromatics or for the screening for microorganisms providing nitroreductase activities. Recently, the reverse reaction, the oxidation of the amine to the nitro group via hydroxylamino and nitroso groups was reported to be catalyzed by p-aminobenzoate oxygenase from Streptomyces thioluteus (Winkler and Hertweck, 2005; Simurdiak et al., 2006). Such arylamine oxygenases might represent interesting resources for the synthesis of new nitro compounds.

In our study, aromatic amine formation took place at relatively low rates as compared to the detected oxidoreductase activities and therefore had only a minor impact on product degradation. Nevertheless, we elucidated the effective inhibition of XMO by aromatic amines in E. coli JM101 (pSPZ3) (Fig. 2.8; Table 2.5). This inhibition apparently takes place on the protein level and in a reversible fashion. The high polarity of aromatic amines suggests a predominant partitioning into the aqueous phase during two-liquid-phase biotransformation. Considering the determined amine formation rates (Table 2.4) and the resulting inhibiting effects (Fig. 2.8; Table 2.5), XMO activity is thought to be completely inhibited about 2 to 3 hours after substrate addition during two-liquid-phase biotransformation, which correlates well with our observations (Fig. 2.4A).
Fig. 2.9. Proposed pattern of enzyme-catalyzed reactions during biotransformation of \textit{m}-nitrotoluene by \textit{E. coli} JM101 (pSPZ3). Vertical direction: XMO catalyzed the multistep oxygenation of \textit{m}-nitrotoluene to the corresponding alcohol (a), aldehyde (b), and acid (c). \textit{m}-Nitrobenzaldehyde formation was counteracted by alcohol dehydrogenase activities of the enzymatic background of the \textit{E. coli} JM101 host, which reduced the aldehyde to the corresponding alcohol (d). Additionally, in \textit{P. putida} DOT-T1E and \textit{P. putida} PpS81, \textit{m}-nitrobenzaldehyde was oxidized to the corresponding acid (e). Horizontal direction: all tested bacterial strains reduced nitroaromatics to aromatic amines (f to i), which strongly inhibited XMO-catalyzed oxidation. The formation of unstable \textit{m}-aminobenzaldehyde by nitroreductases (h) remains unclear.
XMO was active at least four-fold longer when the same experimental setup was used but with pseudocumene instead of m-nitrotoluene as the substrate (Bühler et al., 2002; Bühler et al., 2003a). The prevention of nitroreduction therefore seems to be crucial for efficient XMO-based biotransformation of m-nitrotoluene. Although mutants of E. coli that are deficient in oxygen-insensitive nitroreductases have been identified (McCalla et al., 1978), the suitability of such strains as hosts for process applications remains to be investigated.

Significantly lower rates for aromatic amine formation were detected with P. putida DOT-T1E and P. putida PpS81 than with E. coli JM101 (Table 2.4). This was surprising since P. putida strains are known as effective degraders of many aromatic compounds (Jimenez et al., 2002; Wackett, 2003). However, the slower accumulation of aromatic amines might be explained by the subsequent degradation in P. putida, which may be absent in E. coli (Marvin-Sikkema and de Bont, 1994; Nadeau and Spain, 1995; Zhao and Ward, 2001), although the relatively constant sum of nitroaromatics during incubation of the P. putida strains with m-nitrobenzaldehyde does not support this hypothesis (Fig. 2.5B and C). The generally broad substrate spectra of nitroreductases suggest a lower aromatic amine accumulation rate not only for the tested compounds but also for other nitroaromatics for the P. putida strains under investigation (Schenzle et al., 1999). An increased efficiency of XMO-based biotransformations on various nitroaromatics might therefore be achieved by using P. putida DOT-T1E or P. putida PpS81 instead of E. coli JM101 as the recombinant host strain. Furthermore, the solvent tolerance of P. putida DOT-T1E might be exploited to enlarge the spectrum of organic solvents applicable for in situ product extraction and for the production of a broader range of organic compounds in two-liquid-phase bioreactor setups (de Bont, 1998). m-Nitrotoluene was not oxidized in P. putida strains DOT-T1E and PpS81, yet the synthesis of oxidative enzymes involved in the mineralization of m-nitrotoluene via m-nitrobenzyl alcohol, m-nitrobenzaldehyde, and m-nitrobenzoic acid has been reported for P. putida strain OU83 upon induction with m-nitrotoluene (Ali-Sadat et al., 1995; Walia et al., 2003). Interestingly, m-toluidine accumulated as main product in this strain, apparently without significant inhibition of the m-nitrotoluene hydroxylation.

Effects of dehydrogenases on product formation patterns. The multistep oxygenation of m-nitrotoluene by XMO (Fig. 2.9a to c) was counteracted by host-intrinsic enzyme activities from the E. coli host, which reduced m-nitrobenzaldehyde to m-nitrobenzyl alcohol (Fig. 2.5A; Fig. 2.9d). This reduction has been proposed to be catalyzed by an unspecific alcohol dehydrogenase activity (Bühler et al., 2000; Maruyama et al., 2003). All of m-nitrobenzaldehyde previously formed by XMO was reduced to the corresponding alcohol at
the end of the two-liquid-phase biotransformation (Fig. 2.4A). In contrast to XMO, alcohol dehydrogenase(s) was apparently not inhibited by aromatic amines formed as side-products. A biocatalyst which provides a lower alcohol dehydrogenase activity and a prolonged XMO presence might further increase the efficiency of \textit{m}-nitrobenzyl alcohol accumulation and allow the production of \textit{m}-nitrobenzaldehyde or \textit{m}-nitrobenzoic acid.

\textit{P. putida} PpS81 and \textit{P. putida} DOT-TIE both reduced \textit{m}-nitrobenzaldehyde to \textit{m}-nitrobenzyl alcohol with identical conversion rates (Fig. 2.5B and C; Fig. 2.9d). This suggests that alcohol formation was catalyzed by enzymes different from the alcohol dehydrogenase lacking in \textit{P. putida} PpS81 (Grund \textit{et al.}, 1975). The \textit{m}-nitrobenzaldehyde reduction rates detected in the \textit{P. putida} strains were only half as high as those in \textit{E. coli} JM101 and would therefore counteract the XMO-catalyzed oxidation of \textit{m}-nitrobenzyl alcohol at a significantly lower level in \textit{P. putida} DOT-TIE and \textit{P. putida} PpS81 used as hosts.

However, both \textit{P. putida} strains also oxidized \textit{m}-nitrobenzaldehyde to the corresponding acid (Fig. 2.5B and C; Fig. 2.9e), presumably with an aldehyde dehydrogenase such as NtnC, a \textit{p}-nitrobenzaldehyde dehydrogenase from \textit{Pseudomonas} sp. strain TW3 (James and Williams, 1998). On the other hand, the similar rates of alcohol and acid formation from \textit{m}-nitrobenzaldehyde in \textit{P. putida} DOT-TIE (Fig. 2.5B) point to the activity of a single enzyme that catalyzed both reactions. Such an alcohol dehydrogenase with aldehyde dismutase activity was reported to catalyze the stoichiometric disproportion of acetaldehyde into one equivalent of ethanol and acetic acid in yeast (Trivic \textit{et al.}, 1999). Taking reductive and oxidative activities into account, the net conversion rates on the carbonyl group of \textit{m}-nitrobenzaldehyde were similar in \textit{P. putida} DOT-TIE, \textit{P. putida} PpS81, and \textit{E. coli} JM101, making all of them equally useful as hosts for the production of \textit{m}-nitrobenzaldehyde. Nevertheless, the additional aldehyde dehydrogenase supports XMO-catalyzed oxidation of \textit{m}-nitrobenzaldehyde and makes \textit{P. putida} DOT-TIE and \textit{P. putida} PpS81 interesting as hosts in an XMO-based process for the production of \textit{m}-nitrobenzoic acid from \textit{m}-nitrotoluene. Further studies will be necessary to clarify whether the substrate range is limited to \textit{m}-nitrobenzaldehyde and whether these oxidoreductases are also present in other interesting \textit{P. putida} strains.

\textbf{Effect of enzymatic background activity on cofactor regeneration.} Both the XMO-catalyzed oxidation of \textit{m}-nitrobenzyl alcohol (Fig. 2.9b) (Harayama \textit{et al.}, 1992) and the alcohol dehydrogenase-catalyzed reduction of the resulting \textit{m}-nitrobenzaldehyde in the reverse direction (Fig. 2.9d), require NADH as cofactor. This "futile cycle" might decrease
process efficiency not only by the counteracting product formation but also by limiting NADH availability. No significant cofactor shortage was expected in our setup, since detected \( m \)-nitrobenzaldehyde reduction rates were similar in the two-liquid-phase biotransformations using \( E. \ coli \) JM101 (pSPZ3) (Fig. 2.4A; Fig. 2.6A) and in whole-cell activity assays with \( E. \ coli \) JM101 (Fig. 2.5A). However, this issue may become important for biotransformations with higher and more enduring oxygenase activities (Duetz et al., 2001). Closer identification of the enzymes involved might simplify the generation of knock-out mutants unable to reduce \( m \)-nitrobenzaldehyde to the alcohol. Such an engineered strain might be more efficient as a host for the biocatalytic production of \( m \)-nitrobenzaldehyde or \( m \)-nitrobenzoic acid from \( m \)-nitrotoluene.

Reductions catalyzed by NAD\(^+\)-dependent dehydrogenases are associated with NADH consumption, whereas the oxidations generate NADH. The detected conversion of \( m \)-nitrobenzaldehyde to \( m \)-nitrobenzoic acid in \( P. \ putida \) DOT-TIE and \( P. \ putida \) PpS81 should therefore support cofactor regeneration in an XMO-based process using these strains as hosts. Such an uncoupling from metabolic cofactor regeneration was shown to be useful in synthetic applications with the selective oxidation of 2-methylquinoxaline by recombinant \( E. \ coli \) (G. Ionidis, D. Meyer, and A. Schmid, unpublished data) and might also be relevant for environmental applications such as the biodegradation of \( o \)-chlorotoluene by an engineered \( Pseudomonas \) strain (Haro and de Lorenzo, 2001).

This work confirms that the enzymatic background activity of microbial biocatalysts can interfere with the biotransformation by substrate and product degradation. Side product formation often can be avoided by using recombinant biocatalysts based on host strains such as \( E. \ coli \), which provide a “neutral” enzymatic background due to a narrow catabolic substrate spectrum. We identified host-specific factors limiting the catalytic performance of \( m \)-nitrotoluene biotransformation by XMO in recombinant \( E. \ coli \) JM101. Our results suggest a significantly higher efficiency for the successive oxidation of \( m \)-nitrotoluene to \( m \)-nitrobenzyl alcohol, \( m \)-nitrobenzaldehyde, and \( m \)-nitrobenzoic acid with \( P. \ putida \) DOT-TIE or \( P. \ putida \) PpS81 as the host strain than with \( E. \ coli \) JM101. Future research activities are therefore directed towards the evaluation and development of recombinant \( P. \ putida \) strains for biocatalytic applications.
Acknowledgements

This work was supported by grants from the European Commission (QLK3-2001-00435, acronym BARTOLO).

We are indebted to Bruno Bühler for fruitful discussions, to Andreas Schenzle for helpful advices concerning the detection of aromatic amines, and to Juan L. Ramos for providing *P. putida* DOT-T1E.
Chapter 3: The catalytic efficiency of recombinant *Pseudomonas putida* for the selective oxidation of aromatic and heteroaromatic compounds

Meyer, D., Kuhn, D., Witholt, B., and Schmid, A.
Summary

Extraordinary physiological properties such as solvent tolerance make *Pseudomonas putida* strains interesting candidates as hosts for recombinant biocatalysis in organic syntheses. The stability of biocatalytic performance of such solvent-tolerant recombinants is, however, poorly understood so far. In order to investigate whether oxygenase-catalyzed reactions are influenced, e.g. by solvent tolerance mechanisms, we tested recombinant *P. putida* DOT-TIE, S12, KT2440, and *E. coli* JM101 for the reproducibility of the xylene monooxygenase and styrene monooxygenase catalyzed oxidation of 2-methylquinoxaline to quinoxaline-2-ylmethanol and styrene to styrene oxide, respectively. Significantly higher activity variations were found for biocatalysts based on solvent-tolerant *P. putida* DOT-TIE and S12 as compared to solvent-sensitive *P. putida* KT2440 and *E. coli* JM101. The styrene oxidation rates thereby corresponded to the cellular styrene monooxygenase contents. The higher heterogeneity of gene expression levels of solvent-tolerant as compared to solvent-sensitive strains might represent a general survival strategy to protect against deteriorating environmental conditions. Although maximum specific activities of the solvent-tolerant biocatalysts are promising, the low reproducibility of catalytic performance questions their suitability for routine bioprocess applications.

Introduction

Biotechnological processes demand high product concentrations in order to make downstream processing cost-effective. This is, however, often thwarted by the susceptibility of the microbial biocatalyst to toxic substrates and products. A second, organic liquid phase can be used to extract toxic products from the aqueous phase during bioprocesses and thus to produce higher concentrations of toxic chemicals. Nevertheless, such a setup is limited to the production of chemicals, which preferably partition to non-polar and water-insoluble organic phases that are supported by the producer organisms. During the last decades, various *P. putida* strains have been isolated that are able to grow in the presence of a second phase of polar organic solvents, such as octanol or toluene (Ramos et al., 2002; Sardessai and Bhosle, 2002). The use of such solvent-tolerant strains as biocatalysts in two-liquid-phase biotransformations allows the implementation of more polar organic solvents as extractive phases and thus might increase the range of products that can be synthesized (de Bont, 1998; Sardessai and Bhosle, 2004). Examples are the production of 3-methylcatechol from m-xylene...
or from toluene by recombinant *P. putida* DOT-T1E or *P. putida* MC2 using decanol or octanol as a second liquid phase, respectively (Hüsken et al., 2001b; Rojas et al., 2004), or the production of phenol from glucose by recombinant *P. putida* S12 in the presence of a second phase of octanol (Wierckx et al., 2005).

However, the maximal productivities of these processes are in the order of 0.1 g liter\(^{-1}\) h\(^{-1}\) and thus rather unattractive for industrial applications (see Chapter 1). The low overexpression rates of the genes of interest might represent one reason for the relatively slow product formation, since the recombinant *P. putida* biocatalysts in the mentioned examples encoded only one or a few DNA templates on the chromosome or on stringently regulated catabolic plasmids. The use of multi-copy-number plasmids would increase the number of DNA templates per cell and thus might result in higher enzyme production rates.

In this study, plasmids encoding genes of xylene monooxygenase (XMO) or styrene monooxygenase (SMO) were used to investigate the catalytic performance of various *P. putida* host strains for the selective oxidation of 2-methylquinoxaline to quinoxaline-2-ylmethanol or styrene to styrene oxide, respectively. Recombinant *E. coli* JM101 overproducing XMO and SMO served as a control to evaluate the efficiency of the *P. putida* host strains. These *E. coli* biocatalysts have earlier been used for the production of 3,4-dimethyl benzaldehyde from pseudocumene and enantiopure styrene oxide from styrene (Panke et al., 1999b; Panke et al., 2000; Panke et al., 2002; Bühler et al., 2003b). Biotransformations were performed with resting cells cultivated in the absence of organic solvents. Under these conditions, solvent-tolerant *P. putida* strains might not provide a stable solvent-tolerance phenotype as this could require prior adaptation to sublethal concentrations of organic solvents (Weber et al., 1993; Heipieper and de Bont, 1994; Ramos et al., 1997; Ramos et al., 1998; Neumann et al., 2005; Neumann et al., 2006).

Next to the optimization of induction kinetics, we focused on the stability of monooxygenase activities of solvent-tolerant as compared to solvent-sensitive biocatalysts. This study elucidates and critically discusses limitations for the use of solvent-tolerant *P. putida* strains as biocatalysts in synthetic applications.

**Materials and methods**

**Chemicals.** Chemicals were obtained from Fluka (Buchs, Switzerland) [indole ≥99%; 2-methylquinoxaline, ≥98%; styrene oxide, ≥97%], Aldrich (Buchs, Switzerland) [dicyclopropyl ketone (DCPK), 95%], Merck Schuchardt OHG (Hohenbrunn, Germany)
[styrene >99%], and Acros Organics (Geel, Belgium) [n-octane, >98.5%]. Quinoxaline-2-ylmethanol, quinoxaline-2-carbaldehyde, and quinoxaline-2-carboxylic acid were obtained from Georgios Ioniidis.

**Bacterial strains, plasmids, media, growth conditions, and transformation.** Bacterial strains and plasmids are listed in Table 3.1 and Table 3.2, respectively. Bacteria were grown in Luria-Bertani (LB) complex medium (Difco, Detroit, Mich.), M9 (Sambrook and Russell, 2001), or M9* mineral medium. M9* is identical to M9 mineral medium except a three times higher phosphate salt concentration in order to increase the buffer capacity and the absence of calcium chloride (Panke et al., 1999b). M9 and M9* mineral media were complemented with 1.0 ml/liter USFe trace element solution (Bühler et al., 2003a) and pH was adjusted to 7.4 with 10 M NaOH. *E. coli* and *P. putida* strains were cultivated at 30°C with glucose and citrate (0.5%, wt/vol) as carbon sources, respectively. Citrate served as a carbon source for *P. putida* strains as it does not cause catabolite repression of the $P_{aid}$ promoter encoded on the plasmids that were used in this study (Staijen et al., 1999). Thiamine (10 mg/liter) and antibiotics (kanamycin, 50 mg/liter and gentamycin) were added when appropriate. Gentamycin concentrations were reduced to a minimum in order to maintain selection pressure without impairing cell growth: 20 mg/liter for *E. coli* JM101, *E. coli* DH10B, *P. putida* DOT-T1E, and *P. putida* DOT-T1E-PS34; 30 mg/liter for *P. putida* KT2440; 40 mg/liter for *P. putida* DOT-T1Ecti0 and 60 mg/liter for *P. putida* S12 harboring plasmids with a respective selection marker. 10 mg/liter gentamycin was used for continuous cultivation of *P. putida* DOT-T1E (pTEZ225). *E. coli* strains were transformed with plasmids either by heat-shock (90 seconds at 42°C) after treatment with CaCl$_2$ (Sambrook and Russell, 2001) or by electroporation (Gene Pulser II Electroporator, Bio-Rad Laboratories, Hercules, USA) applying an electric pulse of 4–5 ms with a field strength of 12.5 kV cm$^{-1}$. *P. putida* strains were transformed by electroporation.

**DNA manipulation, constructs, and protein separation.** Restriction nucleases, T4 DNA ligase, and buffers were purchased from Fermentas Life Sciences GmbH (St. Leon-Rot, Germany) except Asp718I endonuclease, which was purchased from Roche Diagnostics GmbH (Hilden, Germany). Plasmid DNA was isolated with QIAprep Spin Miniprep Kit from QIAGen GmbH (Hilden, Germany) according to the protocol of the manufacturer. DNA purification, ligation, and agarose gel electrophoresis were performed according to (Sambrook and Russell, 2001). Sodium dodecylsulfate polyacrylamid gel electrophoresis (SDS-PAGE) was performed according to (Laemmli, 1970).
### Table 3.1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>F′ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara, leu)7697 araD139 galU galK mnpG rpsL κ</td>
<td>(Sambrook and Russell, 2001)</td>
</tr>
<tr>
<td>DH10B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td>thi Δ(lac-proAB) F′(traD36 proAB lacI lacZΔM15)</td>
<td>(Messing, 1979)</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>Rifr, solvent-tolerant</td>
<td>(Ramos et al., 1995)</td>
</tr>
<tr>
<td>DOT-T1E</td>
<td>Rifr, Km r, Sm r, Tel r, tgbB Δ′′ phoA-Kmr tgbD Δ kilAB tgbH Δ Sm, solvent-sensitive mutant of P. putida DOT-T1E with gene deletions in all solvent efflux pump operons</td>
<td>(Rojas et al., 2001)</td>
</tr>
<tr>
<td>DOT-T1E-PS34</td>
<td>Rifr, Km r, solvent-sensitive mutant of P. putida DOT-T1E with deletion of the cis-trans isomerase gene</td>
<td></td>
</tr>
<tr>
<td>DOT-T1Ecti0</td>
<td>Solvent-tolerant</td>
<td>(Junker and Ramos, 1999)</td>
</tr>
<tr>
<td>S12</td>
<td>hsdR1, hsdM r, solvent-sensitive strain</td>
<td>(Hartmans et al., 1990)</td>
</tr>
<tr>
<td>KT2440</td>
<td></td>
<td>(Bayley et al., 1977; Franklin et al., 1981)</td>
</tr>
</tbody>
</table>

Rifr, rifampin; Km r, kanamycin; Sm r, streptomycin; Tel r, telurite

### Table 3.2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCom8</td>
<td>Expression vector for E. coli and Pseudomonas, pMB1 ori, rep, alk regulatory system, Gm r</td>
<td>(Smits et al., 2001)</td>
</tr>
<tr>
<td>pCom10</td>
<td>pCom8, Km r instead of Gm r</td>
<td>(Smits et al., 2001)</td>
</tr>
<tr>
<td>pSPZ3</td>
<td>pBR322-derived, pMB1 ori, encoding xylene monooxygenase genes, xylM and xylA under the control of alk regulatory system, Km r</td>
<td>(Panke et al., 1999b)</td>
</tr>
<tr>
<td>pSPZ10</td>
<td>pBR322-derived, pMB1 ori, encoding styrene monooxygenase genes, styA and styB under the control of alk regulatory system, Km r</td>
<td>(Panke et al., 2000)</td>
</tr>
<tr>
<td>pTEZ225</td>
<td>pCom8, encoding xylene monooxygenase genes xylM and xylA under the control of alk regulatory system, Gm r</td>
<td>This study</td>
</tr>
<tr>
<td>pTEZ240</td>
<td>pCom8, encoding styrene monooxygenase genes styA and styB under the control of the alk regulatory system, Gm r</td>
<td>This study</td>
</tr>
<tr>
<td>pTEZ220</td>
<td>pCom10, encoding xylene monooxygenase genes xylM and xylA under the control of the alk regulatory system, Km r</td>
<td>This study</td>
</tr>
</tbody>
</table>

Km r, kanamycin; Gm r, gentamycin
Plasmid pTEZ225 (10055 bp) encodes the XMO genes \textit{xylM} and \textit{xylA}, under the control of the \textit{alkB} promoter. The genes were extracted from pSPZ3 as an \textit{Asp718I/BamHI} fragment which was inserted into the pCom8 vector digested with the same enzymes via cohesive end ligation. pTEZ220 (10084 bp) was constructed in analogy to pTEZ225 but pCom10 (\textit{Km}) instead of pCom8 (\textit{Gm}) was used as a vector. Plasmid pTEZ240 (9720 bp) encodes the SMO genes \textit{styA} and \textit{styB} under the control of the \textit{alkB} promoter. The genes were extracted from pSPZ10 as a \textit{HindIII/KpnI} fragment, which was inserted into the pCom8 vector digested with the same enzymes via cohesive end ligation. After transformation with the ligation mixtures, \textit{E. coli} DH10B cells harboring correctly ligated plasmids were identified by screening for SMO and XMO catalyzed conversion of indole to indigo, resulting in the formation of blue colonies when grown on LB agar containing 1 mM indole under selection pressure and inducing \textit{n}-octane atmosphere. The identity of the plasmids was confirmed by restriction mapping and DNA sequencing of the inserts.

\textbf{Whole-cell activity assays.} Whole-cell biotransformations with resting cells were used to determine specific XMO and SMO activities for the oxidation of 2-methylquinoxyline to quinoxaline-2-yl-methanol or toluene to benzyl alcohol, benzaldehyde, and benzoic acid and styrene to styrene oxide, respectively. Bacteria were cultivated in LB and M9* before inoculation of 100 ml M9* medium to a cell concentration of 0.02 to 0.03 g cell dry weight (CDW) per liter. Monooxygenase synthesis was induced with 0.025% (vol/vol) DCPK added after 3.25 hours of cultivation in shaking flasks. Cells were harvested after another 3.25 hours of cultivation by centrifugation and resuspended to about 2 g CDW/liter in potassium phosphate buffer (50 mM, pH 7.4) containing glucose (1.0%, wt/vol) or citrate (0.5%, wt/vol) as carbon sources for \textit{E. coli} or \textit{P. putida} strains, respectively. Cell growth rates were calculated based on cell concentrations determined at inoculation, induction, and harvesting (see below).

Portions of 1.0 or 2.0 ml cell suspension were incubated with substrate in sealable pyrex tubes for 10 min at 30°C and 250 rpm in a rotary shaker. Cells were incubated only for 5 min when toluene was used as a substrate. 8.0 mM 2-methylquinoxaline or 1.0 mM toluene served as substrates for XMO and 1.5 mM styrene for SMO. XMO or SMO activities were stopped by acidification with perchloric acid (0.8%, vol/vol) or addition of 0.5 volumes of acetonitrile, respectively. Biotransformations were performed in double for each culture. The cell-free supernatant was analyzed for product formation by reversed phase high performance liquid chromatography (RP-HPLC) (see below) to determine the initial specific product formation
Catalytic performance of recombinant *P. putida* rate, expressed as U/g CDW, whereas one unit corresponded to one µmol product formed per minute.

In order to determine the reproducibility of catalytic performance, typically eight colonies originating from the same transformation were cultivated and used for whole-cell activity assays in parallel. For the determination of induction kinetics and growth inhibition, a culture with exponentially growing bacteria was split and the subcultures were incubated with various DCPK concentrations (0, 0.0025, 0.005, 0.01, 0.0175, 0.025, 0.0375, 0.05, 0.1, and 0.25%, vol/vol) instead of 0.025% (vol/vol) and used for whole-cell activity assays.

**Continuous cultivation.** *P. putida* DOT-T1E (pTEZ225) was cultivated using a lab-scale bioreactor equipment as described earlier (see Chapter 2). A 100-ml overnight culture of *P. putida* DOT-T1E (pTEZ225) grown in M9* mineral medium was used as an inoculum for the bioreactor containing 1.4 liter M9 medium with 0.5% (wt/vol) citrate as the carbon source. Cells were grown in batch mode at 30°C. The pH was maintained constant at 7.4 by regulated addition of phosphoric acid and sodium hydroxide solutions. The stirrer speed and aeration rate were set to 1500 rpm and 1.5 liter air per minute, respectively. Continuous cultivation was started after all citrate was consumed by activating the inflow of M9 medium containing 0.5% (wt/vol) citrate and 10 mg/liter gentamycin. The reactor working volume was kept at 1.5 liter by constant removal of culture liquid. The actual working volume varied between 1.2 and 1.5 liter due to foam formation during cultivation. The initial inflow of medium was adjusted to obtain a preset dilution rate of 0.12 h\(^{-1}\). When steady state was achieved (after at least two reactor volumes of flow through and stable cell concentration) XMO synthesis was induced by addition of DCPK to a final concentration of 0.01% (vol/vol) in the medium and cell broth. Various dilution rates were successively set (0.12, 0.30, 0.06, 0.26, and 0.12 h\(^{-1}\)). At each steady state, about 100 ml of cell suspension was withdrawn and used to determine specific 2-methylquinoxaline hydroxylation rates by whole-cell activity assays (see above).

**Analytical procedures.** 2-Methylquinoxaline, quinoxaline-2-yl-methanol, quinoxaline-2-carbaldehyde, and quinoxaline-2-carboxylic acid were separated by a RP-HPLC system (LaChrom Elite from Merck Hitachi) equipped with a Hypersil ODS C18 column (pore size, 12.5 nm; particle size, 5 µm; inner diameter, 4 mm; length, 125 mm) from Agilent Technologies (Palo Alto, CA, USA). The mobile phase consisted of acetonitrile and water containing 0.1% (vol/vol) perchloric acid. The elution profile was as follows: 5% acetonitrile isocratic over 3 min, followed by 5 to 25% acetonitrile in 7 min and 25 to 100% in 6 min. The flow rate was set to 1.0 ml/min and temperature to 25°C. Detection occurred at a wavelength of 236 nm. Styrene and styrene oxide were separated by the identical RP-HPLC setup, except
for the use of a LiChrospher 60 RP-select B column (pore size, 6.0 nm; particle size, 5 μm; inner diameter, 4 mm; length, 125 mm) from Merck Hitachi. The elution occurred isocratic with 45% acetonitrile and 55% water and detection occurred at 210 nm. Toluene, benzyl alcohol, benzaldehyde, and benzoic acid were separated by a RP-HPLC method developed for the separation of the respective nitro substituted derivatives, which was described elsewhere (see Chapter 2). The cell concentration was determined spectrophotometrically at 450 nm with a spectrophotometer Libra S11 (Biochrom Ltd, Cambridge, U.K.) as described elsewhere (Witholt, 1972). Gravimetical determination of the CDW resulted in 0.17 ± 0.00, 0.21 ± 0.01, and 0.24 ± 0.02 g CDW per absorption unit at 450 nm for cell suspensions of E. coli JM101, P. putida DOT-T1E, and P. putida KT2440, respectively. For P. putida DOT-T1E-PS34 and P. putida DOT-T1Ecti0 the correlation factor of the parental strain P. putida DOT-T1E was applied. For P. putida S12 the correlation factor of P. putida KT2440 was applied. Citrate concentration in samples of the continuous cultivation was determined by an enzymatic test kit (Boehringer Mannheim, R-Biopharm AG, Darmstadt, Germany) according to the protocol of the manufacturer.

Results

Growth and induction kinetics of recombinant P. putida DOT-T1E and E. coli JM101 containing xylene monooxygenase. On plasmid pSPZ3 and pTEZ225 expression of XMO genes (xylMA) is under the control of the alk regulatory system, which can be induced by the addition of alkanes or DCPK to the culture (Grund et al., 1975; Wubbolts, 1994; Bühler et al., 2000). Cultures of exponentially growing bacteria of P. putida DOT-T1E and E. coli JM101 harboring the plasmids pTEZ225 or pSPZ3 were split and subcultures were induced with different concentrations of DCPK. Inhibition of cell growth and the rate of XMO-catalyzed 2-methylquinoxaline oxidation were investigated by whole-cell activity assays (Fig. 3.1). Plasmid pSPZ3 was only tested with E. coli JM101 as the host strain, since it is not stably proliferated in pseudomonads. For each subculture, biotransformations were performed in double, which revealed a relatively high reproducibility of the specific XMO catalyzed oxidation of 2-methylquinoxaline. Quinoxaline-2-yl-methanol was the only detected product for all tested biocatalysts. No further oxidation to quinoxaline-2-carbaldehyde and quinoxaline-2-carboxylic acid was detected by XMO. Apparently, these reactions are exclusively catalyzed by the dehydrogenases of the upper TOL degradation pathway (Wong et al., 2002).
Fig. 3.1. Growth of recombinant *P. putida* DOT-T1E and *E. coli* JM101 and induction kinetics of XMO gene expression. XMO activities for the hydroxylation of 2-methylquinoxaline by *P. putida* DOT-T1E (pTEZ225) (A), *E. coli* JM101 (pTEZ225) (B), and *E. coli* JM101 (pSPZ3) (C) were determined by whole-cell activity assays using various DCPK concentrations for induction. Growth rates were determined based on cell concentrations after 3.25 hours of cultivation in the presence of DCPK.
Maximal specific XMO activities were observed for *P. putida* DOT-T1E (pTEZ225) induced with DCPK concentrations between 0.01% and 0.037% (vol/vol). In contrast, the upper border for maximal activity was 0.1% (vol/vol) DCPK for both *E. coli* JM101 recombinants. Although the pattern of induction kinetics was reproducible, maximally achieved XMO activities varied in repetitions of the experiment especially for *P. putida* DOT-T1E (pTEZ225), which was a subject of further investigations (see below). No residual product formation was detected in the absence of DCPK for *P. putida* DOT-T1E (pTEZ225) and *E. coli* JM101 (pSPZ3) used as biocatalysts, indicating a “tight” regulation of gene expression. A residual XMO activity of 8.1% of maximal activity was found in the absence of DCPK for *E. coli* JM101 (pTEZ225) (data not shown).

Growth of *P. putida* DOT-T1E (pTEZ225) was inhibited by induction already with low DCPK concentrations, whereas growth of *E. coli* JM101 recombinants was significantly reduced for induction with DCPK concentrations higher than 0.05% (vol/vol) (Fig. 3.1). In order to further investigate growth inhibition, the influence of different DCPK concentrations was tested for the wild-type strains not harboring any plasmids and thus not overproducing XMO (Fig. 3.2). Exponential growth could be estimated for DCPK concentrations ≤0.1% (vol/vol). DCPK reduced cell growth rates without being lethal for the cells. In contrast, 0.25% (vol/vol) DCPK completely inhibited growth of *P. putida* DOT-T1E after 2.75 hours of incubation (Fig. 3.2A). A comparison of growth rates showed that *P. putida* DOT-T1E cells not adapted to organic solvents were more sensitive to DCPK than *E. coli* JM101 cells (Fig. 3.2C).

In general, growth rate reductions for the wild-type strains (Fig. 3.2C) were similar as for the corresponding recombinants (Fig. 3.1), suggesting that growth is mainly affected by the inherent toxicity of DCPK rather than by the metabolic burden of XMO overproduction. As an exception, high DCPK concentrations affected growth of *P. putida* DOT-T1E more effectively as compared to *P. putida* DOT-T1E (pTEZ225) (Fig. 3.2C and Fig. 3.1A). This difference might have been the result of inhomogeneous growth behavior of this strain, which was subject of further investigations (see below). Growth rates of *E. coli* JM101 were identical for *E. coli* JM101 (pSPZ3), but systematically higher than for *E. coli* JM101 (pTEZ225). An increased metabolic burden due to a significantly higher plasmid copy number for pTEZ225 than for pSPZ3 in *E. coli* cells might explain this difference (Smits *et al.*, 2001).
For all tested biocatalysts, 0.025% (vol/vol) DCPK was found to result in relatively high specific 2-methylquinoxaline hydroxylation rates. This concentration was therefore used to induce gene expression in all further experiments, although it provoked a slight reduction of P. putida DOT-T1E growth rates.

Reproducibility of the catalytic performance of recombinant P. putida and E. coli strains containing xylene monooxygenase or styrene monooxygenase. Varying maximal specific XMO activities and inhomogeneous growth were observed during the investigation of induction kinetics, especially for P. putida DOT-T1E used as the host strain (see above).
Therefore, we investigated the reproducibility of XMO activity and cell growth in a systematic way by repeated whole-cell activity assays for *P. putida* DOT-TIE (pTEZ225), *E. coli* JM101 (pTEZ225), and *E. coli* JM101 (pSPZ3). Cells from eight different colonies originating from the same transformation plate were cultivated in parallel, induced with 0.025% (vol/vol) DCPK, and tested for the hydroxylation of 2-methylquinoxaline to quinoxaline-2-yl-methanol as well as for the successive oxidation of toluene to benzyl alcohol, benzoic acid, and benzoic acid (Fig. 3.3).

**Fig. 3.3.** Reproducibility of activity and growth of recombinant *P. putida* DOT-TIE and *E. coli* JM101 containing XMO. Left panel: XMO activities of eight cultures used for whole-cell activity assays with 2-methylquinoxaline and toluene as substrates. Right panel: Growth rates before and after induction with 0.025% (vol/vol) DCPK. Tested biocatalysts: *P. putida* DOT-Ti1E (pTEZ225) (A), *E. coli* JM101 (pTEZ225) (B), and *E. coli* JM101 (pSPZ3) (C).
The most significant variation of specific XMO activity was observed for *P. putida* DOT-T1E (pTEZ225) providing a four-fold higher coefficient of variation (the ratio of the standard deviation to the mean activity) than *E. coli* JM101 (pTEZ225) for 2-methylquinoxaline hydroxylation rates (Fig. 3.3 and Table 3.3). The lowest variation of XMO activity was determined for *E. coli* JM101 (pSPZ3).

The specific XMO activities were about two to three-fold higher for the multistep oxidation of the natural substrate toluene as compared to the hydroxylation of 2-methylquinoxaline. The average XMO activity of 103 U/g CDW for the multistep oxidation of toluene by *E. coli* JM101 (pSPZ3) determined in the present study is in accordance with reported activities (100 U/g CDW; (Bühler et al., 2000)), which validates the setup and performance of the whole-cell activity assays for this reproducibility study. Higher deviations observed for the oxidation of toluene than for the oxidation of 2-methylquinoxaline might be explained by an experimental error in product quantification due to the relatively high volatility of toluene and benzaldehyde and from the complexity of the reaction, which involves the successive formation of three products. Therefore, in further experiments 2-methylquinoxaline was used as a routine substrate in whole-cell activity assays.

Maximal specific quinoxaline-2-yl-methanol formation rates were higher for *E. coli* JM101 (pTEZ225) as compared to *E. coli* JM101 (pSPZ3) (Table 3.3). This might be due to a higher plasmid copy number for pTEZ225 as compared to pSPZ3 in *E. coli* hosts. In contrast to plasmid pSPZ3, plasmid pTEZ225 lacks the rop gene, whose product is involved in the regulation of plasmid DNA replication (Cesareni et al., 1982; Smits et al., 2001). Growth of *E. coli* JM101 (pSPZ3) cells was more homogeneous as compared to growth of *E. coli* JM101 (pTEZ225) and *P. putida* DOT-T1E (pTEZ225) cells (Fig. 3.3 and Table 3.3). A higher variation in XMO activity coincided with a higher variation of growth rate. However, no relation could be observed between cell growth rate and the corresponding XMO activity of a clone.

We wanted to investigate whether the variations of activity are also observed for a reaction catalyzed by another monooxygenase such as SMO, which is soluble and thus physiologically different from membrane-bound XMO. Cells harboring plasmid pTEZ240 were analyzed for the SMO-catalyzed oxidation of styrene to styrene oxide in whole-cell activity assays performed in parallel (Fig. 3.4). A relatively high variation of specific SMO activity was observed for *P. putida* DOT-T1E (pTEZ240) providing a coefficient of variation of 43%. In contrast, SMO activity of *E. coli* JM101 (pTEZ240) was more reproducible with a coefficient of variation of 3% (Fig. 3.4 and Table 3.3). Growth of *E. coli* JM101 (pTEZ240) was also
more homogeneous as compared to growth of *P. putida* DOT-TIE (pTEZ240). However, since cells of only three instead of eight colonies of *E. coli* JM101 (pTEZ240) were tested, the statistical significance of activity and growth variation is reduced for this experiment.

Fig. 3.4. Reproducibility of SMO overproduction and activity, and cell growth of recombinant *P. putida* DOT-TIE and *E. coli* JM101. Specific SMO activities for the oxidation of styrene to styrene oxide of three and eight cultures of *E. coli* JM101 (pTEZ240) (A) and *P. putida* DOT-TIE (pTEZ240) (B), respectively, were determined (left panel). Growth rates determined before and after induction with 0.025% (vol/vol) DCPK (right panel). SDS-PAGE of induced *P. putida* DOT-TIE (pTEZ240) cells used for biotransformation in (B) revealing the corresponding StyA contents (at 47 kDa) (C). Identical amounts of biomass were loaded. C, control: non-induced *P. putida* DOT-TIE (pTEZ240) cells; M, protein size marker.
Interestingly, growth rates of *E. coli* JM101 (pTEZ240) were reduced after induction of SMO synthesis with 0.025% DCPK (vol/vol). This DCPK concentration did not significantly inhibit growth of *E. coli* JM101 (pTEZ225) or of the corresponding wild-type strain (Fig. 3.1 and Fig. 3.2). Overproduction of SMO apparently represents a higher metabolic burden for *E. coli* JM101 as compared to overproduction of XMO.

A residual SMO activity of about 50% was observed for non-induced *E. coli* JM101 (pTEZ240), whereas no background activity was detected for *P. putida* DOT-TIE (pTEZ240) (results not shown). The relative high "leakiness" of gene expression determined for both *E. coli* JM101 (pTEZ225) (see above) and *E. coli* JM101 (pTEZ240) coincide with earlier observations with *E. coli* cells harboring a pCom-based plasmid for the overproduction of catechol-2,3-dioxygenase (Smits et al., 2001). Residual catechol oxidizing activity in the absence of inducer was explained by a relative high copy number of the positive regulator protein AlkS in *E. coli* hosts (Smits et al., 2001).

SMO overproduction was visualized by SDS-PAGE. Specific SMO activities of *P. putida* DOT-TIE (pTEZ240) (Fig. 3.4B) corresponded to the cellular SMO contents, estimated based on the intensity of the StyA band on the gel (Fig. 3.4C). StyB was not visible on the gel, presumably due to a lower expression level than for StyA as it was reported for the wild-type strain (Otto et al., 2004).

Significantly higher variations of activity were observed for recombinant *P. putida* DOT-TIE as compared to *E. coli* JM101 irrespective from the overproduced monooxygenase, which, in the case of SMO activity could be referred to varying cellular SMO contents. These results indicate that host specific differences in gene expression, plasmid proliferation, or enzyme stability, rather than in cofactor or substrate availability, caused the differences of activity reproducibility.

Other *P. putida* strains were tested as hosts for XMO and SMO based biocatalysis in order to elucidate whether a high variation of activity is a common feature of all *P. putida* strains. *P. putida* KT2440 is a solvent-sensitive member of the *P. putida* species. Bacteria of this strain are, comparably to *E. coli* cells, highly susceptible to polar organic solvents, such as toluene (Ramos et al., 1998). The genome of *P. putida* KT2440 has been sequenced and degradation pathways have been characterized, which makes this "standard" *P. putida* strain a useful model for studying biochemical, genetic, and biocatalytic aspects (Jimenez et al., 2002; Nelson et al., 2002).

In contrast, *P. putida* S12 cells have solvent efflux pumps and *cis-trans* isomerases similar to those identified in *P. putida* DOT-T1E cells rendering them tolerant against polar organic
solvents (Ramos et al., 2002). *P. putida* S12 was not tested as a host strain for SMO-catalyzed styrene oxide formation since, in contrast to the other tested strains, it is known to degrade styrene (Hartmans et al., 1990).

The variation of activity was significantly lower for *P. putida* KT2440 than for *P. putida* DOT-TIE used as host strains for both XMO and SMO catalyzed reactions (Fig. 3.5A and B and Table 3.3). Regarding XMO activities, the variations for recombinant *P. putida* KT2440 and *E. coli* JM101 were comparable, whereas for *P. putida* S12 variations were similar as for *P. putida* DOT-TIE used as host. Apparently, there is a correlation between solvent tolerance of the host strain and an increased variation of recombinant monooxygenase activity.

For further investigations, solvent-sensitive deletion mutants of *P. putida* DOT-TIE were tested as hosts for the SMO-catalyzed oxidation of styrene to styrene oxide (Fig. 3.5D and E). Variations of SMO activities for both *P. putida* DOT-TIEc0 (pTEZ240), which is deficient for *cis-trans* isomerase, and *P. putida* DOT-TIE-PS34 (pTEZ240), which is deficient for solvent efflux pumps, were comparable to the SMO activity variations of the parental strain used as a host (Table 3.3). Although solvent-tolerant *P. putida* recombinants showed a high variation of activity, solvent tolerance mechanisms were apparently not involved, e.g. by altering the intracellular inducer or substrate concentrations. However, since recombinants based on solvent-tolerant *P. putida* DOT-TIE and *P. putida* S12 were not adapted to organic solvents, their activity of solvent efflux and *cis-trans* isomerization remains unclear.

Growth rates before induction were lower and less reproducible for *P. putida* KT2440 (pTEZ225) as compared to *P. putida* KT2440 (pTEZ240), which might be explained by a longer cell growth lag phase after inoculation (Fig. 3.5A and B). Growth rates before induction of *P. putida* S12 (pTEZ225) tended to correspond with the measured XMO activities (Fig. 3.5C). *P. putida* DOT-TIE-PS34 (pTEZ240) was the slowest growing recombinant strain tested (Table 3.3). The metabolic burden of numerous selection markers (Gm\(^r\), Rif\(^r\), Km\(^r\), Sm\(^r\), and Tef\(^r\)) might have reduced the cell growth rate. Nevertheless, a maximum specific SMO activity of 80.0 U/g CDW was determined in experiments with this biocatalyst, which indicates that activities were rather independent from the growth rate during cultivation.
Fig. 3.5. Reproducibility of activity and growth of various *P. putida* strains containing XMO or SMO. Left panel: Specific XMO or SMO activities of eight cultures of recombinant bacteria for the oxidation of 2-methylquinoxaline or styrene, respectively, after induction with 0.025\%(vol/vol) DCPK. XMO and SMO activities were tested for cells of solvent-sensitive *P. putida* KT2440 harboring plasmids pTEZ225 (A) and pTEZ240 (B), respectively. XMO activity was tested for nonadapted cells of solvent-tolerant *P. putida* S12 (pTEZ225) (C). SMO activity was tested for biocatalysts based on solvent-sensitive mutants of solvent-tolerant *P. putida* DOT-TIE, *P. putida* DOT-TIEcti0 (pTEZ240) (D) and *P. putida* DOT-T1E-PS34 (pTEZ240) (E). Activity of *P. putida* DOT-T1E-PS34 (pTEZ240) culture #7 was not determined since cells failed to grow after induction. Right panel: Growth rates of the cultures before and after induction.
Table 3.3. Summary of growth and catalytic performance in repeated whole-cell activity assays for all tested biocatalysts

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Average growth rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Coeff. of variation&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before induction [h&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>After induction [h&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>Min. [U/g CDW]</td>
<td>Max. [U/g CDW]</td>
</tr>
<tr>
<td><strong>Xylene monooxygenase overproducing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> DOT-TIE (pTEZ225)</td>
<td>0.29 ± 0.06</td>
<td>0.29 ± 0.04</td>
<td>0.4 ± 0.0</td>
<td>45.1 ± 1.7</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-TIE (pTEZ220)</td>
<td>0.32 ± 0.03</td>
<td>0.31 ± 0.05</td>
<td>2.4 ± 0.0</td>
<td>32.8 ± 1.8</td>
</tr>
<tr>
<td><em>P. putida</em> S12 (pTEZ225)</td>
<td>0.39 ± 0.12</td>
<td>0.28 ± 0.04</td>
<td>3.9 ± 0.1</td>
<td>22.5 ± 0.5</td>
</tr>
<tr>
<td><em>P. putida</em> KT2440 (pTEZ225)</td>
<td>0.25 ± 0.05</td>
<td>0.30 ± 0.04</td>
<td>27.9 ± 4.4</td>
<td>45.6 ± 2.4</td>
</tr>
<tr>
<td><em>E. coli</em> JM101 (pTEZ225)</td>
<td>0.32 ± 0.03</td>
<td>0.30 ± 0.05</td>
<td>38.4 ± 1.0</td>
<td>70.5 ± 0.7</td>
</tr>
<tr>
<td><em>E. coli</em> JM101 (pSPZ3)</td>
<td>0.35 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>35.7 ± 1.1</td>
<td>41.9 ± 1.5</td>
</tr>
<tr>
<td><strong>Styrene monooxygenase overproducing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> DOT-TIE (pTEZ240)</td>
<td>0.50 ± 0.05</td>
<td>0.29 ± 0.05</td>
<td>3.9 ± 0.8</td>
<td>80.2 ± 6.5</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-TIE Ecti0 (pTEZ240)</td>
<td>0.42 ± 0.05</td>
<td>0.25 ± 0.08</td>
<td>10.6 ± 0.9</td>
<td>47.5 ± 3.8</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-TIE-PS34 (pTEZ240)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.18 ± 0.02</td>
<td>0.22 ± 0.06</td>
<td>2.9 ± 0.1</td>
<td>80.0 ± 1.7</td>
</tr>
<tr>
<td><em>P. putida</em> KT2440 (pTEZ240)</td>
<td>0.47 ± 0.02</td>
<td>0.32 ± 0.02</td>
<td>6.7 ± 0.4</td>
<td>13.8 ± 2.0</td>
</tr>
<tr>
<td><em>E. coli</em> JM101 (pTEZ240)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.37 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>87.3 ± 2.6</td>
<td>91.6 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Growth rates are based on cell concentrations determined at inoculation, induction with 0.025% (vol/vol) DCPK, and harvesting of the bacteria.

<sup>b</sup> Activities are based on the formation of quinoxaline-2-yl-methanol from 2-methylquinoxaline and styrene oxide from styrene for biocatalysts containing xylene monooxygenase and styrene monooxygenase, respectively.

<sup>c</sup> Coefficient of variation is the ratio of the standard deviation to the mean activity.

<sup>d</sup> Results based on seven instead of eight whole-cell activity assays.

<sup>e</sup> Results based on three instead of eight whole-cell activity assays.
Reasons for the high variation of monooxygenase activity of solvent-tolerant biocatalysts. The variations of specific activities are apparently related to varying cellular enzyme contents, which might be caused for example by differences on the plasmid sequence or by unstable plasmid segregation.

To investigate these possibilities, plasmids were extracted from induced \textit{P. putida} DOT-T1E-PS34 (pTEZ240) cells that were used for whole-cell activity assays (Fig. 3.5E). The linearized plasmids were analyzed by agarose gel electrophoresis, which revealed no visible differences in relative migration or intensity of the DNA fragment bands, indicating constant plasmid size and cellular concentration (data not shown). The regulation of plasmid proliferation apparently was not involved in the observed variation of SMO activity for \textit{P. putida} DOT-T1E-PS34 (pTEZ240) used as a biocatalyst. However, point mutations on the plasmid sequence could not be detected by this method.

The plasmids were used to transform \textit{E. coli} JM101 cells. The coefficient of variation for styrene oxidizing activity of the resulting biocatalysts determined in whole-cell activity assays (Fig. 3.6) was significantly lower (15%) as compared to the \textit{P. putida} DOT-T1E-PS34 recombinants (80%), from which these plasmids were extracted. The specific SMO activities of \textit{P. putida} DOT-T1E-PS34 recombinants did not correlate with activities of \textit{E. coli} JM101 recombinants harboring plasmids from the same origin. It was concluded that the genetic information relevant for expression of SMO genes was not altered on the plasmid, i.e. by mutations, during cultivation and induction in \textit{P. putida} DOT-T1E-PS34 used as a host.

\textit{E. coli} JM101 (pTEZ240)

**Fig. 3.6.** Reproducibility of activity and growth of \textit{E. coli} JM101 harboring plasmid pTEZ240 extracted from recombinant \textit{P. putida} DOT-T1E-PS34. Left panel: SMO activities for the hydroxylation of 2-methylquinoxaline of seven cultures of \textit{E. coli} JM101 harboring plasmid pTEZ240 previously extracted from the respective induced cultures of recombinant \textit{P. putida} DOT-T1E-PS34 (see Fig. 3.5E). Right panel: growth rates before and after induction with 0.025\% (vol/vol) DCPK. n.a., plasmid pTEZ240 from recombinant \textit{P. putida} DOT-T1E-PS34 culture #7 was not available (see Fig. 3.5E).
Gentamycin resistance used as a selection marker can cause problems in the appropriate dosage of gentamycin as well as by a reduction of the growth rate and yield (Smits et al., 2001). To prevent such effects, minimal required gentamycin concentrations were identified and applied for each biocatalyst used in this study (see “Materials and methods”). In addition, the reproducibility of the XMO-catalyzed hydroxylation of 2-methylquinoxaline to quinoxaline-2-yl-methanol by \textit{P. putida} DOT-TIE (pTEZ220) was tested. Plasmid pTEZ220 is identical to pTEZ225 except that it encodes the gene for kanamycin instead of gentamycin resistance. The resulting variation of XMO activity as well as growth were similar for both biocatalysts irrespective of the used selection marker, which excluded gentamycin resistance as a source for the observed activity variations (Fig. 3.7A and Table 3.3).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3_7.png}
\caption{Reproducibility of activity and growth of recombinant \textit{P. putida} DOT-TIE using an alternative antibiotic resistance or inducing agent. (A) Left panel: XMO activities for the hydroxylation of 2-methylquinoxaline of eight cultures of \textit{P. putida} DOT-T1E harboring plasmid pTEZ220, which is identical to pTEZ225 but encodes the gene for kanamycin instead of gentamycin resistance. Right panel: growth rates before and after induction with 0.025\% (vol/vol) DCPK. (B) Left panel: SMO activities of eight cultures of \textit{P. putida} DOT-T1E (pTEZ240) for the oxidation of styrene after induction with 0.1\% (vol/vol) \textit{n}-octane instead of 0.025\% (vol/vol) DCPK. Right panel: growth rates before and after induction.}
\end{figure}
The carbonyl group of DCPK might be reduced by dehydrogenases as it was observed for aromatic aldehydes in *E. coli* and *P. putida* strains (see Chapter 2). The degradation of DCPK used as an inducer could result in an inconsistent monoxygenase synthesis. In order to exclude effects of the inducer on activity variation, DCPK was replaced by *n*-octane for the induction of SMO synthesis in *P. putida* DOT-TIE (pTEZ240). *n*-Octane is a highly efficient inducer of the alk regulatory system, which was earlier investigated for *E. coli* JM101 (pSPZ3) (Bühler et al., 2000). *P. putida* DOT-TIE is unable to grow on *n*-octane, but able to grow on 1-octanol (data not shown), the initial degradation product of *n*-octane metabolizing bacteria (McKenna and Kallio, 1965; Klug and Markovetz, 1971; Smits et al., 2002). It is therefore suggested that *n*-octane used as an inducer is not degraded in *P. putida* DOT-TIE cells.

*P. putida* DOT-TIE (pTEZ240) induced with 0.1% (vol/vol) *n*-octane revealed an average SMO activity of 57.2 ± 28.3 U/g CDW (Fig. 3.7B) as compared to 52.7 ± 22.8 U/g CDW when induced with 0.025% (vol/vol) DCPK (Fig. 3.4B and Table 3.3). The high similarity of catalytic performances suggests that SMO activity variation occurs independent from the agent used for induction.

A single colony of a *P. putida* DOT-TIE (pTEZ225) transformant was spread on solid-medium and eight descending colonies were tested for XMO-catalyzed 2-methylquinoxaline hydroxylation in independent whole-cell activity assays. This procedure ensured monoclonal behavior of all tested clones. The resulting average XMO activity was 17.2 ± 6.4 U/g CDW, which corresponded to a variation coefficient of 37% (Fig. 3.8).

![Fig. 3.8 Reproducibility of activity and growth of *P. putida* DOT-TIE (pTEZ225) originating from the same transformant.](image-url)

Left panel: XMO activities for the hydroxylation of 2-methylquinoxaline. Right panel: Growth rates before and after induction with 0.025% (vol/vol) DCPK.
Thus, although the tested P. putida DOT-TIE (pTEZ225) biocatalysts were genetically identical at the beginning of the experiment, their XMO activities varied significantly. In contrast to the enzyme activities, growth rates were nearly identical (Fig. 3.8). These results show that during cultivation, genetic or physiologic alterations must have taken place, which influenced XMO overproduction and activity but not cell growth of the recombinants.

Xylene monooxygenase activity of P. putida DOT-TIE (pTEZ225) cells grown in continuous cultivation under induced conditions. The cultivation time seems to be an important factor for the variation of activity since it defines the timeframe for alterations to take place in the cells. For whole-cell activity assays, cells were cultivated in shaking flasks for identical time periods. In order to investigate the effects of cultivation time, XMO activity was determined for resting P. putida DOT-TIE (pTEZ225) cells, which have grown in a continuous cultivation under induced conditions (Fig. 3.9A). The DCPK concentration for induction was 0.01% (vol/vol) instead of 0.025% (vol/vol) because it was not possible to obtain a steady state in an earlier continuous cultivation of recombinant E. coli JM101 with maximally induced overexpression of SMO genes under the control of the alk regulatory system (Park, 2004). Medium inflow was adjusted to obtain different dilution rates below the maximal growth rate $\mu_{\text{max}}$ of $0.51 \pm 0.01$ h$^{-1}$ determined during batch cultivation. At each steady state, cell suspension was withdrawn and XMO-catalyzed 2-methylquinoxaline hydroxylation rates were determined in double by whole-cell activity assays.

XMO activity steadily decreased with increasing cultivation time (Fig. 3.9A). Thereby, only a minor XMO activity deviation was determined. The specific XMO activity was not related with the cell growth rate (dilution rate) of the actual steady state. Apparently, the proportion of cells with a reduced or lacking XMO activity increased with increasing cultivation time/total number of generations. Instable plasmid segregation thereby could be excluded as a reason, since the cellular plasmid concentration as well as the approximate plasmid size remained rather constant, as it was determined by agarose gel electrophoresis of the linearized plasmids extracted from cells used for whole-cell activity assays (Fig. 3.9B).

The concentration of induced cells correlated with the dilution rate/growth rate of the actual steady state (Fig. 3.9A). The cultivation took place under carbon limited conditions, indicated by the absence of remaining citrate in the cell broth. The higher cell concentration determined for faster growing cells thus corresponded to a higher biomass yield. This might be due to maintenance energy demands, which reduce biomass yields, especially at low cell growth rates.
Cell concentration was reduced to about 70% after induction with 0.01% (vol/vol) DCPK during cultivation at the preset dilution rate (Fig. 3.9A). The lowered biomass yield could be explained by an increased energy demand due to the overproduction of XMO. Since this DCPK concentration reduced the growth rate of wild-type cells of \textit{P. putida} DOT-T1E (Fig. 3.2), yield reduction could have also been caused by the inherent toxicity of DCPK. However, cell concentration was nearly as high for induced cells in the end as for non-induced cells in the beginning of the continuous cultivation when grown at identical rates. Apparently, the reduction of biomass yield upon induction was compensated during the experiment. This might be explained by the selection of cells with a lower metabolic burden due to a reduced XMO biosynthesis level.

This experiment shows that XMO activity and presumably also XMO synthesis of recombinant \textit{P. putida} DOT-T1E (pTEZ225) not only is varying in repetitions of whole-cell activity assays, but is lost when cells are cultivated for an increased time period under induced conditions.
Fig. 3.9. Continuous cultivation of P. putida DOT-T1E (pTEZ225) containing XMO. (A) Course of cultivation. Medium contained 0.5% (wt/vol) citrate as the carbon source and 10 mg/liter gentamycin. Flow was adjusted to obtain several dilution rates D as indicated in the figure. The arrow indicates the time point of induction with 0.01% (vol/vol) DCPK. Cells were withdrawn at each steady state and used for whole-cell activity assays to determine specific XMO activity for the hydroxylation of 2-methylquinoxaline. No remaining citrate was determined during continuous cultivation in the cell broth. (B) Agarose gel electrophoresis of the linearized plasmids that were extracted from the cells used for biotransformation. Plasmids were linearized by digestion with BsrGI. M, size marker (HindIII digest of λ-DNA); C, linearized control plasmid (pTEZ225 extracted from recombinant E. coli DH10B).
Discussion

The increased variation of activity might be associated with solvent tolerance regulation of the host bacteria. Variations of enzyme activity were relatively high for solvent-tolerant *P. putida* DOT-T1E and *P. putida* S12 but significantly lower for solvent-sensitive *P. putida* KT2440 and *E. coli* JM101 used as hosts (Table 3.3). Although, the reasons for this difference seem to be related to solvent tolerance, a direct influence of the proposed major solvent tolerance mechanisms could be excluded in this study (Fig. 3.5).

The contribution of both mechanisms, cis-trans isomerases and solvent efflux pumps to solvent tolerance is well known. *cis-trans* Isomerases convert *cis*-unsaturated fatty acids of membrane phospholipids to the *trans*-isomers as a major short-term response to organic solvent exposure (Heipieper et al., 1992; Weber et al., 1994; Holtwick et al., 1997; Junker and Ramos, 1999). The more compact packing of lipids consisting of *trans*-unsaturated fatty acids in the lipid bilayer increases the rigidity of the cell envelope and thus compensates for the increased membrane fluidity caused by organic solvents (Heipieper et al., 1992; Sikkema et al., 1995; Heipieper et al., 2003). Solvent efflux pumps extrude organic solvent molecules from the inner bacterial membrane into the extracellular space as an energy-dependent, long-term response of solvent-tolerant bacteria to incubation with organic solvents (Kieboom et al., 1998a; Kieboom et al., 1998b; Ramos et al., 1998; Rojas et al., 2001). Solvent-tolerant *P. putida* strains cultivated in the presence of sublethal concentrations of organic solvents show high viabilities and relatively short growth lag phases upon incubation with a second liquid phase of toxic solvent (Weber et al., 1993; Heipieper and de Bont, 1994; Ramos et al., 1997; Ramos et al., 1998; Neumann et al., 2005; Neumann et al., 2006). In contrast, only a fraction of cells survives such a shock by e.g. toluene, without preexposure (Junker and Ramos, 1999; Duque et al., 2001).

In spite of the detailed characterization of *cis-trans* isomerases and solvent efflux pumps, the regulation of solvent tolerance is still unclear. The inducibility of efflux pump activity indicates that adaptation is an important prerequisite for a strain to develop a stable solvent tolerance phenotype. It is suggested that global regulation factors are activated upon incubation of the cells with organic solvents (Pedrotta and Witholt, 1999; Duque et al., 2001; Ramos et al., 2002). Mutator elements like transposons are one possibility of such regulation factors. Increasing solvent tolerance of *P. putida* S12 was reported to be associated with an increased number of insertion sequences found on the genome, one of them in a putative regulator sequence of solvent efflux pumps (Wery et al., 2001). Apparently, transposons
generate diverse mutations in solvent-tolerant *P. putida* S12, resulting in various subpopulations, which allow a rapid adaptation when confronted with severe adverse conditions (Wery *et al.*, 2001). This might represent a general survival strategy of all solvent-tolerant *P. putida* strains. A culture with subpopulations that provide different levels of recombinant gene expression would explain the high variations of activity observed for biocatalysts based on solvent-tolerant *P. putida* strains. A fraction of cells that less efficiently overproduce XMO and thus have an energetically favorable metabolism might have been selected during continuous cultivation of *P. putida* DOT-T1E (pTEZ225). This would explain the observed loss of ability of the cells to oxidize 2-methylquinolixaline by XMO. However, plasmids extracted from biocatalysts based on solvent-tolerant *P. putida* DOT-T1E and on its solvent efflux pump deficient descendant *P. putida* DOT-T1E-PS34, did not show such mutations. This can be concluded from the constant plasmid size and stable proliferation in *P. putida* strains (Fig. 3.9B) and the regained reproducibility of activity when reintroduced into *E. coli* JM101 as the host strain (Fig. 3.6). Variations in the expression level of plasmid encoded monooxygenase genes could have alternatively been caused by mutations in regulatory sequences on the host genome or as a result of a regulatory phenomenon. Although subpopulations are a plausible explication for the observed heterogeneities, the results of this study do not allow any further conclusions in this respect.

It is possible that the *alk* system, which regulates gene expression on the plasmids used in this study, has an influence on these heterogeneities. Physiological and morphological changes as well as genetic instability of wild-type and recombinant *P. putida* strains were observed upon expression of the *alk* genes for alkane oxidation from *P. putida* GPo1 (Chen *et al.*, 1996). In addition, recombinant *E. coli* W3110 cells, which harbored plasmids encoding the *alk* genes lost their ability to oxidize n-octane to octanoic acid when used as biocatalysts in a two-liquid-phase continuous cultivation with n-octane as the second phase in spite of the presence of antibiotics used for selection pressure (Favre-Bulle *et al.*, 1993). These results are comparable to the loss of XMO activity of *P. putida* DOT-T1E (pTEZ225) cells during continuous cultivation observed in this study (Fig. 3.9). Interestingly, a subpopulation of n-octane-tolerant *E. coli* W3110 wild-type cells was identified that remained viable during the stationary phase of batch cultivation in the presence of a second phase of n-octane (Favre-Bulle *et al.*, 1991; Favre-Bulle and Witholt, 1992). These results confirm the important role of physiological heterogeneities in solvent tolerance, which apparently not only appear in *P. putida* but also in *E. coli* strains. By replacing *E. coli* W3110 with *E. coli* HB101 as a host strain, octanoic acid production remained stable during continuous cultivation (Favre-Bulle *et
This observation correlates with our results of activity variation for *P. putida* KT2440 used as a host, which showed a significantly higher reproducibility as compared to the other host strains of this species. Thus, the stability and reproducibility of the recombinant enzyme synthesis and activity seems to be host strain dependent for both *P. putida* and *E. coli* species. Recently, a homogeneous population for the synthesis of a green fluorescence protein under the control of the *alk* regulatory system in *E. coli* XL10 host cells was determined by flow cytometry (Makart et al., 2006). An extension of this flow cytometry study to other *E. coli* and *P. putida* host strains and the involvement of another expression system might help to further understand the role of subpopulations in solvent tolerance and to evaluate the influence of the *alk* regulatory system on these heterogeneities.

The suitability of solvent-tolerant *P. putida* strains as hosts in recombinant biocatalysis. Maximal specific enzyme activities determined for the tested *P. putida* based biocatalysts were promising with regard to synthetic applications. For example, *P. putida* DOT-T1E (pTEZ240) oxidized styrene to styrene oxide by SMO at a maximal rate of 80 U/g CDW (Table 3.3). This is in the range of specific activities reported for resting cells of *E. coli* JM101 (pSPZ10) (86–92 U/g CDW) containing the same enzyme (Park et al., 2006). This biocatalyst has been used for the production of 388 g enantiopure styrene oxide in a two-liquid-phase pilot-scale biotransformation (Panke et al., 2002). *P. putida* DOT-T1E (pTEZ225) catalyzed the hydroxylation of 2-methylquinoline or the multistep oxidation of toluene with maximal specific XMO activities that were even higher than the activities determined for *E. coli* JM101 (pSPZ3), which was earlier exploited for the production of 469 g 3,4-dimethylbenzaldehyde from pseudocumene (Bühler et al., 2003b).

Nevertheless, *E. coli* JM101 harboring pTEZ225 or pTEZ240 showed higher maximal monooxygenase activities in this study than the corresponding *P. putida* recombinants (Table 3.3). *P. putida*-based biocatalysts thus do not generally provide higher maximal monooxygenase activities than *E. coli*-based biocatalysts. The use of *P. putida* is nevertheless favorable, since expression of pTEZ225 and pTEZ240 encoded monooxygenase genes was "tightly" regulated by the *alk* system in *P. putida* host strains as compared to the corresponding *E. coli* JM101 recombinants, which showed remaining monooxygenase activities in the absence of inducer.

Tuning of gene expression was possible by titration of DCPK for all tested host strains. The pCom vectors thus seem to be useful especially for the construction of *P. putida*-based catalysts not only for biotransformations with catechol-2,3-dioxygenase (Smits et al., 2001) but also with monooxygenases such as XMO or SMO.
However, the low reproducibility of activity of biocatalysts based on solvent-tolerant *P. putida* strains makes them rather unattractive for industrial applications. In addition, we found no means to easily select active clones or to predict their stability, e.g. during a fed-batch biotransformation. Yet, enzyme activities might be more stable for solvent-tolerant bacteria that are adapted to a second phase of octanol or toluene. It is plausible that during adaptation, a subculture is selected, which is able to support the toxic effects of the organic solvents. This selection pressure might result in a more homogeneous culture of bacteria and thus in a more stable enzyme activity in biotransformations. However, the presence of a second phase of octanol or toluene may reduce the biocatalytic performance of solvent-tolerant *P. putida* strains. Proteome analysis indicated that cell metabolism increases energy generation during cultivation in the presence of organic solvents in order to maintain a stable solvent tolerance phenotype, i.e. by active solvent extrusion (Segura *et al.*, 2005; Volkers *et al.*, 2006). Energy demands thus might limit cell growth and, in the case of energy dependent biocatalysis, the biotransformation itself. To circumvent this limitation, the use of an extracting phase such as decanol, which provokes less toxic effects on bacteria, was proposed for two-liquid-phase biotransformations (Rojas *et al.*, 2004; Neumann *et al.*, 2005; Neumann *et al.*, 2006) (see Chapter 4).

The construction of “artificial” solvent-tolerant bacteria by overproducing solvent efflux pumps and *cis-trans* isomerases in a host strain, which is not generating subpopulations, might be a promising solution. Such recombinants would become solvent-tolerant upon induction without prior adaptation and might represent alternative hosts to solvent-tolerant wild-type bacteria for the construction of biocatalysts with an increased reproducibility of activity. Both efficient solvent tolerance and redox biocatalysis are dependent on the metabolic energy generation. Host strains with a higher energy metabolism might therefore be used to construct biocatalysts with a more stable solvent tolerance phenotype and a more efficient biocatalytic performance. As an example, *Paracoccus versutus* regenerates NADH at an estimated rate, which is at least two-fold higher than for *E. coli* or *Pseudomonas* strains (see Chapter 1). Current molecular biology tools would allow broadening the range of bacterial species used as hosts to exploit solvent tolerance in recombinant biocatalysis. However, the success of such “artificial” solvent-tolerant hosts will strongly depend on the clear elucidation of the solvent tolerance phenotype, i.e. identification of the actual contribution of efflux pumps, *cis-trans* isomerases, and other mechanisms to solvent tolerance. In addition, the compatibility of the heterologous solvent tolerance mechanisms
with the host bacterial membranes will be a critical factor, since the membrane is the place where solvent tolerance occurs.

Acknowledgements

This work was supported by grants from the European Commission (QLK3-2001-00435, acronym BARTOLO).

We are indebted to Bruno Bühler for fruitful discussions, to Andreas Meyer for pertinent advices concerning plasmid construction, to Patty Kleinsteuber and Sabine Nussbaum for excellent technical assistance, to Christian Becker (Max-Planck-Institute for Molecular Physiology, D-44202 Dortmund) for providing the electroporator, and to Georgios Ionidis for providing quinoxaline-2-yl-methanol, quinoxaline-2-carbaldehyde, and quinoxaline-2-carboxylic acid as analytical standards.
Chapter 4: *Growth and physiological response of Escherichia coli JM101 in the presence of 1-decanol*

Summary

Effects of 1-decanol on growth and membrane composition of *Escherichia coli* JM101 were investigated. The tested 1-decanol concentrations were not lethal but reduced growth rates of the exponentially growing bacteria in a concentration dependent way. In the presence of a second phase of 1-decanol, growth rate was reduced maximally to about 70% of the rate determined in the absence of 1-decanol. For bacteria that were precultivated with 1-decanol, the same reduced growth rate was determined independently from the tested concentration, which indicates the absence of a long-term adaptation mechanism. On the level of membrane composition, ratio of saturated to unsaturated fatty acids increased with increasing 1-decanol concentrations. This alteration represents a typical short-term bacterial adaptive response to incubation with long-chain alkanols. Contents of phosphatidylethanolamine to phosphatidylglycerol lipids increased when bacteria grew with increasing 1-decanol concentrations, which is the opposite reaction reported for solvent-tolerant *Pseudomonas putida* bacteria incubated with organic solvents. Our results suggest that *E. coli* JM101 cells support a second phase of 1-decanol due to a low cell toxicity of this solvent rather than due to specific solvent tolerance mechanisms. In regard to biocompatibility, this strain is thus suitable as host for two-liquid-phase biotransformations with 1-decanol. The use of *E. coli* JM101 bacteria might even be more favorable than the use of solvent-tolerant bacteria, such as *P. putida* DOT-T1E, which in general need to be adapted in order to be able to grow with a second phase of 1-decanol.

Introduction

The use of bacteria as biocatalysts for the production of value-added chemicals on an industrial scale is attracting increasing interest (Schmid et al., 2001a). The presence of a second liquid phase during biotransformation is often useful to increase production yields and biocatalyst stability by separating the aqueous medium containing the bacteria from toxic substrates and products, which accumulate in the second phase (Schmid et al., 1998; Bühler and Schmid, 2004). These second liquid phases are typically unpolar, water-immiscible, and thus not cell toxic organic solvents, such as dioctylphthalate or *n*-hexadecane (see Chapter 1 and 5). However, their extraction potential for compounds with an intermediate polarity is limited. Extraction properties of more polar organic solvents such as alcohols like 1-octanol, 1-nonanol, and 1-decanol might be more effective for the production of e.g. catechols or
Physiological response of *E. coli* to 1-decanol

toluene. Yet, the relatively high cell toxicity of these organic solvents makes their use critical in biotransformations with “normal” bacteria. Therefore, exclusively solvent-tolerant bacteria, such as *P. putida* DOT-TIE have been used as hosts for such biotransformations, so far (Rojas et al., 2004; Neumann et al., 2005; Neumann et al., 2006). *P. putida* DOT-TIE bacteria can be adapted to grow in a second phase of cell toxic organic solvents by a stepwise increase of the solvent concentration during cultivation (Weber et al., 1993; Heipieper and de Bont, 1994; Ramos et al., 1995). However, adaptation procedures for 1-octanol and 1-nonanol are protracting and growth rates in the presence of a second phase are significantly reduced. In contrast, solvent-tolerant *P. putida* DOT-TIE bacteria were shown to easily adapt to 1-decanol resulting in relatively high growth rates after adaptation. Therefore 1-decanol was proposed to be the optimal second phase for two-liquid-phase biotransformations with solvent-tolerant bacteria (Rojas et al., 2004; Neumann et al., 2005; Neumann et al., 2006).

In general, cell toxicity of an organic solvent is associated with its polarity, which can be experimentally determined by the partitioning of the chemical in an octanol–water two-phase system (Seeman, 1972; Sikkema et al., 1995; Weber and de Bont, 1996). Organic solvents with a logarithm of the partition coefficient (logP<sub>ow</sub>) between 1 and 4 provide a relatively high aqueous solubility and preferably partition into the lipid bilayer, which leads to their accumulation to high concentrations in membranes when present as a second phase. This provokes an increase of the fluidity of the bacterial membrane and impairs its structure and functions, which prevents “normal” bacteria from growing and ultimately can lead to cell death (Laane et al., 1987; Weber and de Bont, 1996). 1-Decanol has a logP<sub>ow</sub> of 4.0 (Laane et al., 1987) and thus is on the edge of being toxic for bacteria that are not solvent-tolerant. *E. coli* JM101 is a bacterial strain that has been successfully used as recombinant host for a number of biotransformations with or without an unpolar second phase (Held et al., 1998; Held et al., 1999; Panke et al., 1999b; Panke et al., 2000; Schmid et al., 2001b; Bühler et al., 2002; Panke et al., 2002; Bühler et al., 2003a; Bühler et al., 2003b; Meyer et al., 2003; Yildirim et al., 2004; Yildirim et al., 2005; Park et al., 2006; Yildirim et al., 2006) (see also Chapter 2, 3, and 5). Here, growth and physiological response of *E. coli* JM101 to the presence of different concentrations of 1-decanol was investigated in order to evaluate whether this strain is a useful candidate for two-liquid-phase biotransformations with 1-decanol.
Materials and methods

**Chemicals, bacterial strain, and media.** Chemicals were purchased with highest purity from Fluka (Buchs, Switzerland) or Sigma-Aldrich (Steinheim, Germany). Acetonitrile and methanol (LC-MS grade) were purchased from Carl Roth (Karlsruhe, Germany). *E. coli* JM101 (supE thi Δ(lac-proAB) F’(traD36 proAB’ lacF' lacZΔM15)) was used in this study (Messing, 1979) and was cultivated in Luria-Bertani (LB) complex medium (Difco, Detroit, Mich.), M9* mineral medium (Panke et al, 1999b), or in a mineral medium as described by (Hartmans et al., 1989). M9* is identical to M9 mineral medium (Sambrook et al., 1989) except that it contained three times higher phosphate salt concentrations to increase the buffer capacity and did not contain calcium chloride (Panke et al., 1999b). M9* was supplemented with 1 ml/liter USFe trace element solution (Bühler et al., 2003a), 10 mg/liter thiamine, and 0.5% (wt/vol) glucose. The pH was adjusted to 7.4 with 10 M NaOH solution. The Hartmans mineral medium was supplemented with 10 mg/liter thiamine and contained 0.4% succinic acid (wt/vol) as the carbon source. The pH was adjusted to 7.0 with 10 M NaOH solution.

**Growth conditions and determination of cell concentration.** Bacteria were cultivated in baffled 250-ml-shaking flasks containing 50 ml mineral medium in a rotary shaker at 30°C and 180 rpm after successive precultivation in LB and mineral medium. For analysis of growth rate as well as membrane phospholipid and fatty acid compositions bacteria were cultivated for 3 to 3.5 hours in Hartmans mineral medium before different amounts of 1-decanol were added as dilutions (30 μl) in acetone (final 1-decanol concentrations: 0, 0.037, 0.074, 0.111, 0.148, 0.185, 0.222, 0.260, or 0.297 mM). A negative control was cultivated in the absence of 1-decanol and acetone. The shaking flasks were sealed in order to avoid evaporation of 1-decanol. When indicated, bacteria were precultivated in the presence of successively increased 1-decanol concentrations in mineral medium until saturation was obtained (0.3 mM 1-decanol). This culture was then used to inoculate mineral medium containing different concentrations of 1-decanol (final 1-decanol concentrations: 0.034, 0.070, 0.106, 0.141, 0.177, 0.213, 0.249, 0.284, or 0.320 mM added as dilutions in acetone). For investigating effects of a bulk amount of 1-decanol on growth, bacteria were cultivated for 3 hours in 50 ml M9* mineral medium before addition of 1-decanol (0, 10, or 50 ml). Shaking frequency was increased to 250 rpm after 1-decanol addition in order to allow the formation of an emulsion.

Growth of bacteria was monitored during cultivation by spectrophotometrical determination of cell concentration at a wavelength of 450 nm as described elsewhere.
Physiological response of E. coli to 1-decanol (Witholt, 1972). When necessary, phases were allowed to separate before determination of cell concentration in the aqueous phase.

**Lipid extraction and transesterification, analysis of fatty acid methyl esters by gas chromatography via flame ionization detection (GC-FID).** Exponentially growing bacteria in the presence of different concentrations of 1-decanol were harvested, washed with a phosphate buffer (50 mM, pH 7.0) and lipids were extracted by a procedure adapted to (Bligh and Dyer, 1959). Bacteria (about 15 mg dry weight) resuspended in 0.5 ml H2O were extracted with 1 ml methanol and 1.75 ml chloroform by vigorous shaking for about 3 min followed by the addition of 0.5 ml H2O and shaking for 30 sec. After separation of the phases by centrifugation, the chloroform phase containing the lipids was removed and dried. Fatty acid methyl esters (FAME) were prepared according to (Morrison and Smith, 1964) by incubation of the lipids with boron trifluoride-methanol and extraction with n-hexane. FAME were detected by GC-FID as described by (Neumann et al., 2005).

**Analysis of lipids by liquid chromatography-mass spectrometry (LC-MS).** Lipids recovered from the chloroform phase before transesterification (see above) were solved in methanol and diluted in a 4:1 (vol/vol) mixture of acetonitrile and an aqueous buffer (5 mM ammonium acetate, pH 4.8) prior to analysis.

Chromatographic separations were performed using a Surveyor MS pump and Surveyor autosampler (Thermo Electron, San Jose, CA, USA). The injection volume was set to 2 µl. A base deactivated Discovery Bio Wide Pore C-5 column from Supelco (Deisenhofen, Germany) was used as stationary phase (pore size, 300 Å; particle size, 3 µm; inner diameter, 1.0 mm; length, 100 mm). Flow rate of the mobile phase was 85 µl/min. A binary gradient consisting of acetonitrile and aqueous buffer (5 mM ammonium acetate, pH 4.8) was used for elution: 45% acetonitrile isocratic for 2 min, followed by 45 to 85% in 34 min, from 85 to 100% in 6 min, 100% isocratic for 15 min, from 100 to 45% in 3 min, and then 45% acetonitrile isocratic for 15 min.

Mass spectrometric detection was carried out using a LTQ FT Fourier transform ion cyclotron resonance hybrid mass spectrometer (Thermo Electron, Bremen, Germany), equipped with a 7.0 Tesla actively shielded superconducting magnet and electrospray ionization source. The instrument was operated in the data-dependent mode. Briefly, the LTQ FT was set to automatically switch between MS and MS/MS acquisition. Survey MS spectra in the mass range m/z 300–1000 were acquired in the FTICR in the negative ionization mode with a resolution power r = 100,000. The five most intense ions were sequentially isolated for fragmentation in the linear ion trap by collisionally induced dissociation. The total cycle time
was approximately 3 s. The general mass spectrometric conditions were: spray voltage, 3.0 kV; sheath gas flow, 35 arb units; auxiliary gas flow, 4 arb units; sweep gas flow, 2 arb units; ion transfer tube temperature, 250°C; and normalized collision energy 30% for MS/MS with activation $q = 0.25$ and activation time 30 ms. Ion selection thresholds were 500 counts for MS/MS.

Results

**Growth and membrane fatty acid composition of E. coli JM101 in the presence of different concentrations of 1-decanol.** Exponentially growing *E. coli* JM101 cells were incubated with different concentrations of 1-decanol to test the effects of this organic solvent on cell growth. Bacteria that were not incubated with 1-decanol during precultivation grew with reduced rates in the presence of 1-decanol in a concentration-dependent manner (Fig. 4.1A). At saturating 1-decanol concentration (>0.23 mM), bacteria grew with about 70% of the rate determined without 1-decanol addition. The use of acetone as a solvent for 1-decanol did not have significant effects on cell growth. Rates of $0.29 \pm 0.01$ h$^{-1}$ or $0.28 \pm 0.02$ h$^{-1}$ resulted with or without acetone, respectively. Bacteria that were precultivated with 1-decanol grew at similar rates, independently from the tested 1-decanol concentration. The lack of growth rate regeneration in spite of bacteria grew for about four generations with a reduced 1-decanol concentration, indicates the presence of a relatively stable adaptive response to this organic solvent. However, a long-term adaptation that occurred during precultivation could be excluded since growth rates of bacteria precultivated with 1-decanol were similar as the rates of not 1-decanol precultivated bacteria incubated with saturating 1-decanol concentrations.

Bacteria can respond with an alteration of the membrane fatty acid composition to changing environmental conditions, such as the incubation with organic solvents. We therefore extracted membrane lipids from the *E. coli* JM101 bacteria used in the growth experiments and determined the membrane fatty acid compositions by GC-FID. Membrane lipids consisted mainly of palmitic (16:0), cis-palmitoleic (16:1 cis), and cis-vaccenic acids (18:1 cis). Minor components were myristic (14:0), stearic (18:0), and 9,10-methylene-hexadecanoic acids (17 cyc) (Table 4.1). No trans-unsaturated fatty acids were detected.
Fig. 4.1. Growth and membrane fatty acid saturation of *E. coli* JM101 incubated with different concentrations of 1-decanol. Different amounts of 1-decanol were added to exponentially growing bacteria in Hartmans medium with 0.4% (wt/vol) succinic acid as the carbon source (with or without precultivation of the bacteria with 1-decanol). (A) Growth rates were determined after addition of 1-decanol. (B) The ratios of the saturated to the corresponding unsaturated fatty acids were determined in the membranes of the cells harvested at the end of the growth experiment (see Table 4.1). Fatty acid composition but not the growth rate was determined for bacteria in the presence of a bulk amount of 1-decanol (volume ratio, medium/decanol = 5/1; corresponds to about 900 mM 1-decanol). The dashed vertical line indicates the maximal aqueous solubility of 1-decanol at 25°C (0.234 mM) (Barton, 1984).
Whereas membrane contents of 16:0 remained relatively constant, 18:0 contents increased with increasing 1-decanol concentration, which coincided with a decrease of unsaturated fatty acid contents (16:1 \textit{cis} and 18:1 \textit{cis}). Thus, the ratio of saturated to unsaturated fatty acids increased in a concentration-dependent way with its maximum for bacteria grown with a bulk amount of 1-decanol (Fig. 4.1B). The degree of fatty acid saturation was higher for bacteria that have been precultivated with 1-decanol as compared to not preincubated bacteria.

### Table 4.1: Effects of 1-decanol on the membrane fatty acid composition of \textit{E. coli} JM101

<table>
<thead>
<tr>
<th>Precultivation conc. [mM]</th>
<th>1-decanol conc.</th>
<th>Fatty acid contents(^a)</th>
<th>Degree of saturation(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/o decanol</td>
<td></td>
<td>14:0 16:0 16:1 \textit{cis} 17 cyc 18:0 18:1 \textit{cis}</td>
<td></td>
</tr>
<tr>
<td>0(^c)</td>
<td>0.8</td>
<td>37.2 21.1 1.5 2.7 34.9</td>
<td>0.71</td>
</tr>
<tr>
<td>0</td>
<td>0.8</td>
<td>37.3 22.2 1.3 2.4 33.9</td>
<td>0.71</td>
</tr>
<tr>
<td>0.037</td>
<td>0.8</td>
<td>37.6 20.5 1.2 3.1 34.8</td>
<td>0.74</td>
</tr>
<tr>
<td>0.074</td>
<td>0.7</td>
<td>37.7 20.2 1.3 2.8 35.4</td>
<td>0.73</td>
</tr>
<tr>
<td>0.111</td>
<td>0.7</td>
<td>37.6 20.1 1.2 2.8 35.5</td>
<td>0.73</td>
</tr>
<tr>
<td>0.148</td>
<td>0.8</td>
<td>37.7 20.9 1.2 2.5 34.8</td>
<td>0.72</td>
</tr>
<tr>
<td>0.185</td>
<td>0.7</td>
<td>37.6 20.2 1.2 2.9 35.2</td>
<td>0.73</td>
</tr>
<tr>
<td>0.222</td>
<td>0.7</td>
<td>38.0 18.9 1.1 3.1 36.0</td>
<td>0.75</td>
</tr>
<tr>
<td>0.259</td>
<td>0.8</td>
<td>38.3 18.9 1.1 3.6 35.3</td>
<td>0.77</td>
</tr>
<tr>
<td>0.297</td>
<td>0.8</td>
<td>37.9 19.2 1.1 3.4 35.6</td>
<td>0.75</td>
</tr>
<tr>
<td>Sec. phase(^d)</td>
<td>1.2</td>
<td>36.5 18.4 0.9 8.7 32.1</td>
<td>0.90</td>
</tr>
<tr>
<td>With decanol(^e)</td>
<td></td>
<td>14:0 16:0 16:1 \textit{cis} 17 cyc 18:0 18:1 \textit{cis}</td>
<td></td>
</tr>
<tr>
<td>0.034</td>
<td>0.8</td>
<td>37.5 20.0 1.2 3.8 34.6</td>
<td>0.76</td>
</tr>
<tr>
<td>0.070</td>
<td>0.8</td>
<td>37.7 19.9 1.2 3.8 34.6</td>
<td>0.76</td>
</tr>
<tr>
<td>0.106</td>
<td>0.8</td>
<td>38.0 19.4 1.2 4.2 34.3</td>
<td>0.79</td>
</tr>
<tr>
<td>0.141</td>
<td>0.8</td>
<td>37.8 19.2 1.2 4.6 34.2</td>
<td>0.79</td>
</tr>
<tr>
<td>0.177</td>
<td>0.8</td>
<td>37.7 18.9 1.2 5.0 34.3</td>
<td>0.80</td>
</tr>
<tr>
<td>0.213</td>
<td>0.9</td>
<td>38.0 19.2 1.2 4.8 34.0</td>
<td>0.80</td>
</tr>
<tr>
<td>0.249</td>
<td>0.9</td>
<td>38.0 18.8 1.1 4.8 34.4</td>
<td>0.81</td>
</tr>
<tr>
<td>0.284</td>
<td>0.9</td>
<td>38.0 18.8 1.1 5.8 33.5</td>
<td>0.84</td>
</tr>
<tr>
<td>0.320</td>
<td>0.9</td>
<td>37.9 18.7 1.1 5.5 34.0</td>
<td>0.83</td>
</tr>
<tr>
<td>Sec. phase(^d)</td>
<td>1.3</td>
<td>36.9 16.5 0.9 11.3 31.1</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Abbreviations: 14:0, myristic acid; 16:0, palmitic acid; 16:1 \textit{cis}, \textit{cis}-palmitoleic acid; 17 cyc, 9,10-methylenehexadecanoic acid; 18:0, stearic acid; 18:1 \textit{cis}, \textit{cis}-vaccenic acid.

Samples with underlined data were also analyzed for lipid composition (see Table 4.2)

\(^a\) Fatty acids were detected by GC-FID and contents are expressed as percentage (wt/wt) of the total fatty acid content.

\(^b\) Sum of 16:0 and 18:0 contents divided by the sum of 16:1 and 18:1 contents.

\(^c\) Negative control without acetone as solvent for 1-decanol

\(^d\) The 50-ml culture was incubated with 10 ml 1-decanol.

\(^e\) Precultivation with successively increasing 1-decanol concentrations (see “Materials and methods” for details).
Membrane lipid composition of *E. coli* JM101 grown in the presence of different concentrations of 1-decanol. Lipid compositions of *E. coli* JM101 grown either in the absence of 1-decanol, with a saturated 1-decanol concentration (about 0.3 mM), or with a second phase of 1-decanol were determined by LC-MS. As a negative control, a culture without acetone as solvent for 1-decanol was analyzed. More than 90% of the sample material was identified as phospholipids with either a phosphatidylethanolamine (PE) or a phosphatidyglycerol (PG) head group (Table 4.2). Also small amounts of diphosphatidylglycerol / cardiolipin (CL) phospholipids were detected. CL lipids comprise only about 1.8 mol% of the total phospholipid content of *E. coli* (Koppelman et al., 2001) and were therefore not taken into consideration in this analysis. All detected unsaturated fatty acid residues contained only one carbon double bond. No free fatty acids were detected in the samples. Alterations in the phospholipid composition as a result to incubation of the bacteria with 1-decanol were in general more pronounced for bacteria that have been precultivated with 1-decanol as compared to bacteria precultivated in the absence of 1-decanol. The ratio of PE over PG lipid contents increased for *E. coli* JM101 grown with increasing 1-decanol concentrations (Fig. 4.2). PE and PG phospholipids that were present at more than 5% of the total are presented in Fig. 4.3A and B, respectively. The PE lipids with molecular weights (MW) of 689.50 u and 717.53 u represented about 50 to 60% of the total PE lipids. The membrane content of the lighter lipid decreased whereas the content of the heavier lipid increased in bacteria growing with increasing 1-decanol concentrations. Both lipids contained one saturated and one unsaturated fatty acid residue. The changing contents of these lipids in the membrane therefore did not have a significant effect on the overall saturation degree as it was observed in the GC-FID analysis of the fatty acid residues (Fig. 4.1B).
Table 4.2. Effects of 1-decanol on the membrane lipid composition of *E. coli* JM101

<table>
<thead>
<tr>
<th>Head group</th>
<th>Mol. weight [u]</th>
<th>Formula</th>
<th>Precultivation without 1-decanol</th>
<th>Precultivation with 1-decanol</th>
<th>Fatty acid residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipid contents</td>
<td></td>
<td>No dec.</td>
<td>No dec.</td>
<td>0.297</td>
</tr>
</tbody>
</table>
Physiological response of *E. coli* to 1-decanol

<table>
<thead>
<tr>
<th>Lipid</th>
<th>722.51</th>
<th>734.51</th>
<th>746.51</th>
<th>748.53</th>
<th>760.53</th>
<th>762.54</th>
<th>774.54</th>
<th>776.56</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{38}H_{75}O_{10}P</td>
<td>0.13</td>
<td>2.52</td>
<td>10.5</td>
<td>43.6</td>
<td>0.57</td>
<td>1.52</td>
<td>21.7</td>
<td>0.51</td>
</tr>
<tr>
<td>C_{39}H_{75}O_{10}P</td>
<td>0.11</td>
<td>2.52</td>
<td>10.1</td>
<td>44.5</td>
<td>0.56</td>
<td>1.48</td>
<td>21.9</td>
<td>0.50</td>
</tr>
<tr>
<td>C_{40}H_{75}O_{10}P</td>
<td>0.30</td>
<td>2.08</td>
<td>6.88</td>
<td>51.0</td>
<td>0.42</td>
<td>2.19</td>
<td>20.5</td>
<td>1.11</td>
</tr>
<tr>
<td>C_{41}H_{76}O_{10}P</td>
<td>0.29</td>
<td>1.93</td>
<td>6.65</td>
<td>49.8</td>
<td>0.39</td>
<td>2.69</td>
<td>20.5</td>
<td>1.16</td>
</tr>
<tr>
<td>C_{42}H_{75}O_{10}P</td>
<td>0.21</td>
<td>2.21</td>
<td>7.43</td>
<td>51.5</td>
<td>0.45</td>
<td>2.00</td>
<td>21.9</td>
<td>0.99</td>
</tr>
<tr>
<td>C_{43}H_{76}O_{10}P</td>
<td>0.25</td>
<td>2.02</td>
<td>6.47</td>
<td>52.5</td>
<td>0.32</td>
<td>2.50</td>
<td>19.9</td>
<td>1.26</td>
</tr>
<tr>
<td>C_{44}H_{77}O_{10}P</td>
<td>0.27</td>
<td>1.87</td>
<td>6.01</td>
<td>52.5</td>
<td>0.33</td>
<td>2.57</td>
<td>20.7</td>
<td>1.48</td>
</tr>
</tbody>
</table>

Samples were analyzed 2–6 times. Standard deviations of lipid contents were below 0.001%.

a Lipids were detected by LC-MS and their contents were expressed as percentage of total PE or PG lipid peak areas.

b Precultivation with successively increasing 1-decanol concentrations (see “Materials and methods” for details).

c Phospholipid head group: PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

d Negative control without acetone as solvent for 1-decanol

e The 50-ml culture was incubated with 10 ml 1-decanol.

f The different fatty acid residue combinations determined by LC-MS for each lipid. The identification of the chemical composition of the residues allowed determining the length of their carbon chains as well as their degree of saturation.
Fig. 4.2. Phospholipid composition of *E. coli* JM101 grown in the presence of different concentrations of 1-decanol. Different amounts of 1-decanol were added to exponentially growing bacteria in Hartmans medium with 0.4% (wt/vol) succinic acid as the carbon source (with or without precultivation of the bacteria with 1-decanol). The membrane lipid composition was determined from bacteria that were harvested at the end of the growth experiment (see Fig. 4.1A). Lipids with phosphatidylglycerol (PG) or phosphatidylethanolamine (PE) head groups were detected. Their relative contents and the chemical structures of the head group residues are shown in the figure. Detected traces of phospholipids with cardiolipin (CL) head group were not taken into account. The asterisk denotes that the sample contained no acetone as a solvent for 1-decanol.

The PG lipid with a MW of 748.53 u was the most abundant phospholipid detected in the membrane of *E. coli* JM101 representing about 50% of the total PG lipids and up to 30% of the total phospholipid content. Its membrane content increased for bacteria grown with increasing 1-decanol concentration (Fig. 4.3B). In all detected fatty acid combinations, this lipid contained a saturated and an unsaturated residue with varying chain lengths between 14 and 20 carbon atoms (Table 4.2).

A higher number of different fatty acid residues was detected by LC-MS as compared to the detection by GC-FID. However, by LC-MS only the chemical composition of the residues could be elucidated. Identification of their structure would require further investigations, such as GC-MS analysis of the respective FAME and comparison with comprehensive standards.
Physiological response of *E. coli* to 1-decanol

**A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>MW (u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precultivation without</td>
<td>MW = 689.50</td>
</tr>
<tr>
<td>1-decanol</td>
<td>MW = 703.52</td>
</tr>
<tr>
<td>Precultivation with</td>
<td>MW = 715.52</td>
</tr>
<tr>
<td>1-decanol</td>
<td>MW = 717.53</td>
</tr>
<tr>
<td></td>
<td>MW = 743.55</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Condition</th>
<th>MW (u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precultivation without</td>
<td>MW = 720.49</td>
</tr>
<tr>
<td>1-decanol</td>
<td>MW = 746.51</td>
</tr>
<tr>
<td>Precultivation with</td>
<td>MW = 748.53</td>
</tr>
<tr>
<td>1-decanol</td>
<td>MW = 774.54</td>
</tr>
</tbody>
</table>

**Fig. 4.3. Contents of the major phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) lipids in *E. coli* JM101 grown in the presence of different concentrations of 1-decanol.** Different amounts of 1-decanol were added to exponentially growing bacteria in Hartmans medium with 0.4% (wt/vol) succinic acid as the carbon source (with or without precultivation of the bacteria with 1-decanol). The membrane lipid composition was determined from bacteria that were harvested at the end of the growth experiment (see Fig. 4.1A) The figure shows phospholipids that represented more than 5% of the total of PE or PG lipid contents (see Table 4.2). The asterisk denotes that cultivation occurred without acetone as a solvent for 1-decanol.
Due to the same chemical formula of 17:1 and 17 cyc, it is likely that 17:1, which was detected by LC-MS, is not a heptadecenoic acid residue but represents 17 cyc (9,10-methylenehexadecanoic acid), whose structure was identified by GC-FID. Other fatty acid residues with odd chain lengths might represent cyclopropyl-containing residues or residues with branched chains. The exploitation of this novel mass spectrometry based methodology might be useful to further investigate the adaptation of bacteria to growth in the presence of organic solvents on the level of membrane alterations.

The lipids with MWs of 687.48 u, 689.50 u, and 691.52 u contained two unsaturated, one unsaturated and one saturated, and two saturated fatty acid residues, respectively (Table 4.2). Fig. 4.4 shows the proportions of the three lipids as a function of the 1-decanol concentration during growth of the bacteria. Thereby the proportion of the double unsaturated lipid (MW, 687.48 u) decreased whereas the proportion of the completely saturated lipid (MW, 691.52 u) increased with increasing 1-decanol concentrations. These results reflect the increase of saturation degree of fatty acids as a function of the alkanol concentration, which was observed by the analysis of the fatty acid residues by GC-FID (Fig. 4.1B).
Fig. 4.4. Proportions of three related PE lipids with fatty acid residues containing none, one, or two carbon double bonds of *E. coli* JM101 grown in the presence of different 1-decanol concentrations. Different amounts of 1-decanol were added to exponentially growing bacteria in Hartmans medium with 0.4% (wt/vol) succinic acid as the carbon source (with or without precultivation of the bacteria with 1-decanol). The membrane lipid composition was determined from bacteria that were harvested at the end of the growth experiment (see Fig. 4.1A). The proportion of the three PE lipids is expressed as the percentage of their total content (see Table 4.2). The asterisk denotes that cultivation occurred without acetone as a solvent for 1-decanol.

**Growth of *E. coli* JM101 in the presence of a second phase of 1-decanol.** Exponentially growing *E. coli* JM101 bacteria (not precultivated with 1-decanol) were incubated with bulk amounts of 1-decanol in order to determine whether a second phase of this organic solvent additionally influences cell growth. After 1-decanol addition, bacteria continued to grow exponentially with 70 to 80% of the growth rate determined without 1-decanol (Fig. 4.5). After the carbon source was depleted, cell concentration rapidly decreased independently from the presence of 1-decanol. This might be explained by an increased susceptibility of the bacteria to shear forces from the vigorous shaking (250 rpm) when entering the stationary growth phase. Although the tested cultures contained the same initial glucose concentration, the rapid initiation of the decline phase observed in this experiment does not allow the determination of biomass yields from the maximally achieved cell concentrations.
Fig. 4.5. Growth of *E. coli* JM101 in the presence of a second phase of 1-decanol. 50-ml cultures with exponentially growing cells (M9*, 0.5% (wt/vol) glucose, pH 7.4, 30°C, 250 rpm) were either incubated with 10 ml (squares) or 50 ml (triangles) of 1-decanol. The time point of 1-decanol addition is indicated by an arrow. As a control, no 1-decanol was added to the culture (diamonds). Growth rates were determined during exponential growth phase after 1-decanol addition (based on cell concentrations represented by the filled symbols).

The same relative growth rate reduction was observed for bacteria incubated with saturating concentrations (Fig. 4.1A) or a bulk amount of 1-decanol (Fig. 4.5), although absolute rates were different due to the use of different media and carbon sources. In addition, the rates were similar for a decanol/medium ratio of 1/5 and 1/1 (Fig. 4.5). These results indicate that growth was not further influenced by the presence or by the volumetric extent of a second phase of 1-decanol. In contrast to the identical relative growth rate reductions, a significantly higher membrane saturation degree was detected for bacteria that grew in the presence of a second phase as compared to saturating 1-decanol concentrations (Fig. 4.1B). This difference might be explained by a direct interaction of the bacteria with the 1-decanol phase, including, e.g., the extraction of lipids or fatty acid residues from the bacterial membrane.
Discussion

Comparison of growth and membrane composition of *E. coli* JM101 and solvent-tolerant *P. putida* strains in the presence of 1-decanol. Bacteria can compensate the increase of membrane fluidity provoked by organic solvents via alterations of the membrane composition. The increase of saturated over unsaturated membrane fatty acids, as it was observed for *E. coli* JM101 when incubated with 1-decanol, thereby represents a typical response of various bacteria to incubation with long-chain alkanols (Ingram, 1976; Heipieper and de Bont, 1994; Kabelitz *et al.*, 2003). The increase of the membrane saturation thereby is lower for bacteria incubated with 1-decanol as compared to incubation with more polar and thus more cell toxic alkanols, such as 1-octanol or 1-nonanol (Sullivan *et al.*, 1979; Kabelitz *et al.*, 2003). Apparently, the intensity of response correlates with the cell toxicity of the organic solvent. The membrane saturation degree increased from 0.71 to 0.77 for *E. coli* JM101 (Fig. 4.1B and Table 4.1) as compared to 0.9 to 1.2 for *Acinetobacter calcoaceticus* when incubated with saturating 1-decanol concentrations (Kabelitz *et al.*, 2003). Under these conditions, *E. coli* JM101 grew with about 70% of the growth rate determined in the absence of 1-decanol, whereas growth of *A. calcoaceticus* was completely inhibited (Kabelitz *et al.*, 2003). These results indicate a correlation between the intensity of response and the susceptibility of the microorganism to a certain organic solvent. The relatively small increase of the fatty acid saturation degree determined for *E. coli* JM101 and the absence of fatty acid alterations reported for *E. coli* ML308 when incubated with 1-decanol (Sullivan *et al.*, 1979) suggest a relatively high tolerance of *E. coli* strains to this alkanol. Indeed, growth experiments on solid agar overlaid with different aliphatic and aromatic organic solvents revealed that *E. coli* has a higher solvent tolerance as compared to other bacterial strains (Inoue and Horikoshi, 1991; Rajagopal, 1996).

Besides an increase of the saturation degree, solvent-tolerant *P. putida* strains also convert cis to trans unsaturated fatty acids of membrane lipids as a short-term response when incubated with organic solvents (Heipieper and de Bont, 1994; Weber *et al.*, 1994). Trans-unsaturated fatty acids can be packed more compactly in the lipid bilayer, which increases the rigidity of the membrane and reduces its fluidity (Heipieper *et al.*, 1992; Sikkema *et al.*, 1995; Heipieper *et al.*, 2003). Nevertheless, an increase of the trans-unsaturated fatty acid content did not enable *P. putida* DOT-T1E bacteria to grow with saturating 1-decanol concentrations (Neumann *et al.*, 2005). In contrast, *E. coli* bacteria, which are unable to synthesize trans-fatty acids (Holtwick *et al.*, 1997), were able to grow in the presence of a second
1-decanol phase without adaptation (Fig. 4.5). These results question the relevance of cis-trans isomerization for solvent tolerance of *P. putida* strains (see also Chapter 1).

Solvent-tolerant *P. putida* cells alter the membrane composition in the presence of organic solvents also on the level of the lipid head group (Ramos et al., 2002). Incorporation of lipids with relatively small head groups and low melting temperatures, such as PE is reduced and incorporation of lipids with larger head groups and higher melting temperatures, such as PG or CL is increased to rigidify the membrane and to reduce its fluidity (Weber and de Bont, 1996; Ramos et al., 2002). As an example, the ratio of the PE over the PG content decreased for *P. putida* DOT-T1E cultivated in the presence of *n*-heptane, *n*-propylbenzene, or toluene (Ramos et al., 1997). Our lipid analysis, however, showed the opposite response when *E. coli* JM101 grew in the presence of increasing 1-decanol concentrations. Apparently, alterations of the lipid composition of *E. coli* JM101 on the level of the head group are not directly associated with an increase of tolerance to 1-decanol. The lipid head group composition thus might be only of minor importance for solvent tolerance in general.

As a long-term adaptation, solvent-tolerant *P. putida* strains synthesize efflux pumps when incubated with sublethal concentrations of organic solvents. The active extrusion of solvent molecules from the inner membrane into the extracellular space ultimately enables these strains to grow with a second phase of cell toxic organic solvents, such as toluene (logP<sub>ow</sub>, 2.5) (Kieboom et al., 1998a; Kieboom et al., 1998b; Ramos et al., 1998; Rojas et al., 2001). Adapted *P. putida* DOT-T1E bacteria grow with about 90% of the rate determined in the absence of 1-decanol whereas non-adapted bacteria are unable to grow with saturating 1-decanol concentrations (Rojas et al., 2004; Neumann et al., 2005; Neumann et al., 2006). The growth rate of *E. coli* JM101 was not recovered during precultivation with 1-decanol, which indicated the absence of such a long-term adaptation for this strain (Fig. 4.1A). Nevertheless, the reduced growth rate in the presence of 1-decanol points to the involvement of an energy-dependent solvent tolerance mechanism, such as efflux pumps, which are coupled to the proton motive force. Indeed, variants of an *E. coli* K12 strain, which were obtained as spontaneous or chemically generated mutants, showed an increased tolerance to organic solvents such as *p*-xylene (logP<sub>ow</sub>, 3.1) during cultivation in liquid medium (Aono et al., 1991), which was (at least partly) ascribed to the activity of multidrug exporters (Aono, 1998; Hayashi et al., 2003). These exporters are closely related to the solvent efflux pumps in *P. putida* strains since both variants belong to the resistance nodulation division type (Ramos et al., 1998; Murakami and Yamaguchi, 2003). The feasibility to increase solvent tolerance by mutagenesis suggests a relatively simple regulation of solvent efflux activity in these *E. coli*
Physiological response of *E. coli* to 1-decanol

K12 strains. The long-term adaptation of solvent-tolerant *P. putida* strains points to a more complex regulation, such as a change in the metabolism or in the enzymatic constitution. *E. coli* JM101 is a descendant of *E. coli* K12 and thus provides the genotypic prerequisite for the reported multidrug efflux system (Messing, 1979; Yanisch-Perron et al., 1985). A swifter activation of solvent efflux due to a relatively simple regulation would explain the absence of long-term adaptation for *E. coli* JM101. Nevertheless, further studies will be necessary to prove the involvement of a multidrug efflux system in the tolerance of *E. coli* JM101 to organic solvents, such as 1-decanol.

**The usefulness of *E. coli* strains as hosts in two-liquid-phase biotransformations with 1-decanol as the second phase.** We showed that *E. coli* JM101 grows at a relatively high rate during cultivation on mineral medium in the presence of a second phase of 1-decanol without the need for a long-term adaptation (Fig. 4.5). These results characterize *E. coli* JM101 as a promising host strain candidate for two-liquid-phase biotransformations with 1-decanol being even more favorable as e.g. solvent-tolerant *P. putida* DOT-T1E, for which cells require a protracting adaptation phase in order to grow under such conditions. In addition, *E. coli* W3110 bacteria are able to grow in a mineral medium culture containing a second phase of *n*-heptane, which has the same logP<sub>ow</sub> as 1-decanol (logP<sub>ow</sub>, 4.0) and thus is supposed to cause similar cell toxic effects (Favre-Bulle et al., 1991). Solvent-tolerant mutant *E. coli* OST3410 containing recombinant phenol hydroxylase was used as biocatalyst for the production of indigo from indole in the presence of a second phase of diphenylmethane (logP<sub>ow</sub>, 4.2) (Doukyu et al., 2003). Productivities thereby were more than four-fold higher than by using the parental strain as host. Although the diphenylmethane phase was not lethal for bacteria of the parental *E. coli* strain, the authors explained the enhanced indigo production with the higher viability of the mutants under reaction conditions. These examples show the feasibility and the general potential of *E. coli* strains used in two-liquid phase biotransformations with organic solvents having a polarity comparable to 1-decanol.

The solvent tolerance potential of *E. coli* strains is not as high as compared to solvent-tolerant *P. putida* strains, of which adapted cells are able to grow in liquid complex medium containing a second phase of toluene (logP<sub>ow</sub>, 2.5) or styrene (logP<sub>ow</sub>, 2.9) (Ramos et al., 1995). Bacteria belonging to the genera of *Klebsiella* and *Serratia* were reported to be as tolerant as *E. coli* and grew on solid media when overlaid with organic solvents such cyclohexane (logP<sub>ow</sub> 3.4) (Inoue and Horikoshi, 1991). These results indicate that also other Gram-negative bacteria might be useful as host strains in two-liquid-phase biotransformations with 1-decanol, although bacteria in general support a second phase of organic solvents with
lower logP_{ow} during cultivation on solid than in liquid media. In addition, other organic solvents with logP_{ow} below four, such as cyclohexane (logP_{ow}, 3.4) or propylbenzene (logP_{ow}, 3.8) might serve as potential second phases in two-liquid-phase biotransformations. However, the usefulness of an organic solvent for two-liquid-phase biotransformations is not only evaluated based on the biocompatibility of the solvent but also with regard to extraction properties and practical handling (see Chapter 1). 1-Decanol (logP_{ow}, 4.0) has a significantly higher polarity than commonly used second liquid phases, such as dioctylphthalate (logP_{ow}, 9.6) or n-hexadecane (logP_{ow}, 8.8). This suggests a better extraction of organic chemicals with high or intermediate polarity, such as small aromatic or aliphatic compounds by 1-decanol. Nevertheless, aromatic compounds might be extracted more efficiently by an aromatic solvent, such as dioctylphthalate due to the chemical similarity of the solute and the solvent (Panke et al., 2000). Thus, the need of replacement of unpolar organic solvents by 1-decanol for extraction of industrially relevant compounds has to be critically investigated. In addition, practical properties such as chemical, biological, and thermal stability, costs and availability in bulk amounts, and toxicity for both operator and environment have to be considered for the further evaluation of 1-decanol as second phase for two-liquid-phase biotransformations (Daugulis, 1988; Bruce and Daugulis, 1991).

Acknowledgements

This work was supported by grants from the European Commission (QLK3-2001-00435, acronym BARTOLO). We are indebted to Helma Geltenpoth (Institute for Analytical Sciences, D-44139 Dortmund) for excellent interpretation of the LC-MS data.
Chapter 5: Towards the production of (R)-phenylacetylcarbinol by the multistep biotransformation of toluene coupled to the central metabolism of *Escherichia coli*

Meyer, D., Kehl, P., and Schmid, A.
Summary

A new whole-cell approach for the production of (R)-phenylacetylcarbinol ((R)-PAC) from toluene with Escherichia coli overproducing xylene monooxygenase (XMO) and an engineered pyruvate decarboxylase (PDCW392M) was developed. XMO catalyzed the successive oxidation of toluene to benzyl alcohol and benzaldehyde, which then was condensed with pyruvate to (R)-PAC catalyzed by PDCW392M. The host strain not only synthesized heterologous enzymes but also regenerated cofactors and provided pyruvate as cosubstrate via the central carbon metabolism. In biotransformations with different resting E. coli-based biocatalysts and crude extracts thereof, the accumulation of benzaldehyde to relatively high concentrations was elucidated as a prerequisite for efficient (R)-PAC production by PDCW392M due to the low affinity of the enzyme for this substrate. Engineering of cultivation conditions and toluene feed in single-phase and two-liquid-phase biotransformations on liter-scale allowed a more enduring production and stabilization of benzaldehyde against oxidation and reduction to benzoic acid and benzyl alcohol by XMO and native dehydrogenases, respectively. Effects on (R)-PAC formation were discussed. Although the (R)-PAC productivities obtained in this study cannot compete with current production processes, major bottlenecks were identified that now can be addressed for further optimization of the process.

Introduction

(R)-PAC is the precursor for the chemical synthesis of ephedrine and pseudoephedrine, which are widely used in pharmaceutical preparations. The traditional method for the production of (R)-PAC is the enzymatically catalyzed decarboxylation of pyruvate followed by the condensation of the product with added benzaldehyde to (R)-PAC (Neuberg and Hirsch, 1921). Both decarboxylation and carboligation are catalyzed by pyruvate decarboxylase (PDC) as major and minor activities, respectively. PDC occurs in various species, e.g., of plants and microbes (Pohl, 1997). Enzyme activity thereby is dependent on the cofactors thiamine pyrophosphate and magnesium ions, which strongly stabilize the quaternary structure of the PDC homotetramer (Diefenbach and Duggleby, 1991; Pohl et al., 1994). A wide range of microorganisms are useful for (R)-PAC production, such as yeasts (Saccharomyces, Schizosaccharomyces, Candida, Nadsonia or Hansemula) (Netrval and Vojtisek, 1982; Rosche et al., 2003), filamentous fungi (Rhizopus javanicus) (Oliver et al.,
1999; Rosche et al., 2001), and bacteria (Zymomonas mobilis and Zymobacter palmae) (Bringer-Meyer and Sahm, 1988; Rosche et al., 2003). With yeast cells, maximally achieved (R)-PAC concentrations are in the range of 10–12 g/liter with yields of 55–60% based on benzaldehyde (Rogers et al., 1997). (R)-PAC production by whole microbes has the advantage of endogenous pyruvate generation from the carbon source via the central carbon metabolism while pyruvate has to be supplied when purified PDC is used as a biocatalyst (Oliver et al., 1999). On the other hand, benzaldehyde is reduced to benzyl alcohol in whole cells by native oxidoreductase activities (e.g. alcohol dehydrogenases), which do not interfere in cell-free extracts due to the lack of electron donors (e.g. NADH) (Rosche et al., 2001).

Amino acid sequence of PDC from Z. mobilis was altered by site-directed mutagenesis of the corresponding gene (Pohl, 1997). Cell-free biotransformation of benzaldehyde to (R)-PAC was best performed with the mutant enzyme PDCW392M due to an increased activity and stability as compared to the wild-type enzyme. The enantiomeric excess thereby was greater than 98% (Pohl, 1997; Goetz et al., 2001). In a biphasic aqueous–pentanol emulsion an overall concentration of 9.0 g/liter (R)-PAC was obtained in 72 hours of biotransformation with isolated PDCW392M enzymes (Rosche et al., 2004).

In this work, we evaluate a new route for the production of (R)-PAC from toluene instead of the more expensive benzaldehyde as substrate by E. coli recombinants containing the Z. mobilis PDC mutein PDCW392M and xylene monooxygenase (XMO) from Pseudomonas putida mt-2 (Fig. 5.1). Benzaldehyde as a substrate for PDC thereby is provided by the successively catalyzed oxidation of toluene to benzyl alcohol and benzaldehyde by XMO. In addition, XMO activity was evaluated for the usefulness to counteract the reduction of benzaldehyde to benzyl alcohol catalyzed by native enzymes of the E. coli host.
Materials and methods

Chemicals, bacterial strains, plasmids, media, growth conditions, and transformation. Chemicals were purchased with highest purity from Fluka (Buchs, Switzerland). (R)-PAC was obtained with technical purity from Michael Breuer (BASF AG, Ludwigshafen, Germany). Bacterial strains and plasmids are listed in Table 5.1. Bacteria were grown in Luria-Bertani (LB) complex medium (Difco, Detroit, Mich.), M9* mineral medium (identical to M9 mineral medium (Sambrook et al., 1989) except that it contained three times more phosphate salts in order to increase the buffer capacity and did not contain calcium chloride) (Panke et al., 1999b), and RB mineral medium (Bühler et al., 2003a). Both mineral media were
supplemented with 1 ml/liter USFe trace element solution (Bühler et al., 2003a). Antibiotics (ampicillin, 50 mg/liter; chloramphenicol, 30 mg/liter; kanamycin, 50 mg/liter), thiamine (10 mg/liter), and leucine (100 mg/liter) were added when appropriate. The pH of the M9* mineral medium was adjusted to 7.4 with 10 M NaOH before use in shaking flasks.

<table>
<thead>
<tr>
<th>Table 5.1. Bacterial strains and plasmids used in this study</th>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli K-12</td>
<td>DH10B</td>
<td>F’ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoK Δ(ara, leu)7697 araD139 galU galK mupG rpsL. λ’</td>
<td>(Sambrook and Russell, 2001)</td>
</tr>
<tr>
<td></td>
<td>JM101</td>
<td>thi Δ(lac-proAB) F’(traD36 proAB+ lacI’ lacZΔM15)</td>
<td>(Messing, 1979)</td>
</tr>
<tr>
<td>Escherichia coli B KO20</td>
<td>prototroph, pfl:(pdc+ adhB+)a, high-level expressing adhB mutant.</td>
<td>(Ohta et al., 1991)</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td>pSPZ3</td>
<td>pBR322-derived, pMB1 ori, encoding XMO genes, xyfM and xyfA under control of the alk regulatory system, Km’</td>
<td>(Panke et al., 1999b)</td>
</tr>
<tr>
<td></td>
<td>pPDC-W392M-His6</td>
<td>pBTac2-derived, pMB1 ori, encoding the mutated pyruvate decarboxylase gene of PDCW392M with 6 His codons at 3’ end. tac promoter, Amp’</td>
<td>(Bruhn et al., 1995; Bruhn et al., 1996; Pohl, 1997)</td>
</tr>
<tr>
<td></td>
<td>pPYKec</td>
<td>pTrc99a-derived, pMB1 ori, lacI’’, encoding pykF’ the gene of the major pyruvate kinase isoenzyme of E. coli, trc promoter, Amp’</td>
<td>(Emmerling et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>pCK01</td>
<td>low-copy number vector, pSC101 oriV, lacZa, Cm’</td>
<td>(Fernandez et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>pTEZ210</td>
<td>pCK01-derived, pSC101 oriV, encoding the mutated pyruvate decarboxylase gene of PDCW392M with 6 His codons at 3’ end. lac promoter, Cm’</td>
<td>This study</td>
</tr>
</tbody>
</table>

Km, kanamycin; Amp, ampicillin; Cm, chloramphenicol

An artificial operon of genes of the alcohol dehydrogenase II (adhB) and pyruvate decarboxylase (pdc) from Z. mobilis were introduced in the pyruvate formate-lyase (pfl) gene region of the E. coli chromosome.

Cultivations were performed at 30°C, and glucose (0.5%, wt/vol) was used as a carbon source. Bacteria were transformed with plasmids either by heat-shock (90 seconds at 42°C) after treatment with CaCl2 or by electroporation applying an electric pulse of 4–5 ms with a field strength of 12.5 kVcm⁻¹ (Sambrook and Russell, 2001). For the construction of
recombinants that simultaneously harbored plasmids pSPZ3 and pTEZ225, transformation with one plasmid was followed by transformation of a resulting recombinant with the second plasmid.

**DNA manipulation and constructs.** Restriction nucleases, T4 DNA ligase, and buffers were purchased from Roche Diagnostics GmbH (St. Leon-Rot, Germany). Plasmid DNA was isolated with High Pure Plasmid Isolation Kits from Roche Diagnostics GmbH (St. Leon-Rot, Germany) according to the protocol of the manufacturer. DNA purification, ligation, and agarose gel electrophoresis were performed according to (Sambrook and Russell, 2001).

Plasmid pTEZ210 (5375 bp) is a derivative of the vector pCK01 encoding the engineered PDCW392M gene (Pohl, 1997) (Table 5.1). The gene was extracted from plasmid pPDC-W392M-His\(_6\) as an EcoRI/HindIII fragment (including the shine-dalgarno sequence at the 5' end and the His tag sequence at the 3' end) and was inserted via cohesive end ligation into the pUC18 polylinker of pCK01 digested with the same enzymes. After transformation with the ligation mixtures, *E. coli* DH10B cells harboring correctly ligated plasmids were selected by α-complementation (Sambrook et al., 1989). The plasmid identity was checked by restriction mapping and DNA sequencing of the insert.

**Whole-cell activity assays.** Bacteria were grown in shaking flasks containing M9* medium and synthesis of the enzymes of interest was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) (for PDC and PykF) and \(n\)-octane (for XMO) added to final concentrations of 0.2 mM and 0.1% (vol/vol), respectively. After four hours of cultivation under induced conditions, the bacteria were harvested and resuspended in potassium phosphate buffer (50 mM, pH 6.5) containing 0.5% (wt/vol) glucose, 50 mg/liter thiamine pyrophosphate, and 5 mM MgSO\(_4\). For biotransformation on ml-scale, the cell suspension was incubated with different concentrations of substrates in Eppendorf tubes on a thermomixer at 25°C and 1400 rpm. Reactions were stopped after different time points by heat deactivation at 100°C for two minutes. All biotransformations were performed at least twice. After centrifugation, the supernatant was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) (see below). Specific activities were expressed in units per gram cell dry weight (U/g CDW). One unit is defined as the enzyme activity that forms one μmol of product per minute.

A modified protocol was used for biotransformations with *E. coli* DH10B (pSPZ3, pTEZ210) recombinants, which were resuspended in potassium phosphate buffer (50 mM, pH 7.0) containing only 1.0% (wt/vol) glucose. The cell suspension was incubated with substrates in sealable Pyrex tubes on a horizontal shaker at 30°C and 200 rpm. Product
formation was monitored by stopping the reaction at different time points by acidification with 40 µl/ml of a 10% (vol/vol) perchloric acid solution.

**Crude extract activity assays.** Bacteria were cultivated and induced as described for whole-cell activity assays. After being harvested, bacteria were washed and resuspended in potassium phosphate buffer (50 mM, pH 6.5) containing 50 mg/liter thiamine pyrophosphate, and 5 mM MgSO₄. The bacteria were disrupted by means of a French press SIM AMINCO and cell debris was removed by centrifugation (GSA rotor, 4000 rpm, 4°C, 30 min). The supernatant represented the crude extract, which was used for biotransformations as described for whole-cell activity assays (see above). The protein concentration of the crude extract was determined according to (Bradford, 1976) using bovine serum albumin for calibration. Sodium dodecylsulfate polyacrylamid gel electrophoresis (SDS-PAGE) was performed according to (Laemmli, 1970).

**Biotransformations on liter scale.** The used bioreactor equipment was earlier described by (Wubbolts et al., 1996) (see Chapter 2 and 3). It had a total volume capacity of 2.6 liter and was used for both single-phase and two-liquid-phase biotransformations. A 100-ml overnight culture of *E. coli* JM101 (pSPZ3, pTEZ210) in RB medium was used as inoculum for a bioreactor containing 1.4 liter RB medium with 0.7% (wt/vol) glucose as the carbon source. For the two-liquid-phase setup, the reactor contained only 0.9 liter medium. For the biotransformation with n-hexadecane as second liquid phase, a reactor equipment KLF2000 from Bioengineering (Wald, Switzerland) having a total volume capacity of 3.1 liter was used with 0.7% (wt/vol) glycerol instead of glucose as the carbon source. Cells grew in batch mode at 30°C overnight. The pH was maintained constant at 7.4 by regulated addition of phosphoric acid and ammonium hydroxide. The stirrer speed and aeration rate were set to 1500 rpm and 1 liter air per minute, respectively. Fed-batch cultivation was started by activating the feed of a solution containing 730 g glucose or 600 g glycerol and 19.6 g or 8.1 g MgSO₄·7 H₂O per liter, respectively, after complete consumption of the carbon source. XMO synthesis was fully or partly induced by the addition of dicyclopropyl ketone (DCPK) to a concentration of 0.02% (vol/vol) or 0.002% (vol/vol), respectively, in the aqueous phase. PDCW392M synthesis was fully induced with 0.2 mM IPTG. After cultivation for two to three hours under induced conditions, biotransformation was started by the addition of toluene as the substrate (represents time zero) either directly as repeated pulses (320 µl every 30 min), continuously as a 25% (vol/vol) solution in ethanol with a peristaltic pump (initial rate of 0.4 ml solution/min), or as part of the second liquid phase. One liter bis(2-ethylhexyl)phthalate (BEHP) or 300 ml n-hexadecane served as carrier solvents and were added to one liter or
800 ml cell broth, respectively. The n-hexadecane phase contained 240 mmol toluene, whereas 100 mmol toluene was added one hour after BEHP addition. The organic phases contained 60 mM n-octane to ensure full XMO induction during two-liquid-phase biotransformations. For the biotransformation with BEHP, XMO synthesis was not induced in the aqueous phase whereas PDCW392M synthesis was induced already during the batch cultivation. The stirrer speed and aeration rate were increased to 2000 rpm and 2 liters per minute, respectively, in the presence of the second liquid phase. After separation of the aqueous and organic phases of samples by centrifugation, substrate and products were detected by RP-HPLC and gas chromatography (GC) (see below). Acetic acid concentrations in the aqueous phase were determined by GC as described elsewhere (Bühler et al., 2003a). Glucose and glycerol concentrations were determined by enzymatic kits from Seil Diagnostics GmbH (Martinsried, Germany) according to the protocol of the manufacturer. Samples from the single phase biotransformations and from the aqueous phase of the two-liquid-phase biotransformation with n-hexadecane were extracted with an equal volume of bis(2-ethylhexyl)phthalate prior to glucose or glycerol quantification in order to reduce the concentration of aromatic substrate and products that might have influenced the analysis. The cell concentration was determined spectrophotometrically at 450 nm as described elsewhere (Witholt, 1972). Dissolved oxygen tension (pO₂) was expressed as the percentage of the tension determined under maximal aeration and stirring in the absence of bacteria.

**Determination of toxicities and partition coefficients of toluene and its metabolites.** Inhibition of growth of *E. coli* JM101 by toluene, benzyl alcohol, benzaldehyde, benzoic acid, and (R)-PAC was determined by a procedure adapted to (Bühler et al., 2002). A culture consisting of exponentially growing bacteria in complete M9* medium was subdivided into sealable shaking flasks and the different compounds were added to concentrations between 0 and 40 mM. Incubation was continued and growth rates were determined based on cell density measurements (data not shown). Toxin concentrations at which the growth rate was reduced to the half of the maximal growth rate (half-inhibiting concentrations) and at which growth was completely inhibited (critical concentrations) were estimated. Partition coefficients were determined as described elsewhere (Bühler et al., 2003a).

**Analytical procedures.** Toluene, benzyl alcohol, benzaldehyde, benzoic acid, and (R)-PAC in aqueous samples were separated with an RP-HPLC instrument (LaChrom Elite from Merck Hitachi) equipped with a Nucleosil C18 column (pore size, 100 Å; particle size, 5 μm; inner diameter, 4 mm; length, 25 cm) from Macherey-Nagel (Oensingen, Switzerland). The mobile phase consisted of acetonitrile and H₂O containing 0.1% perchloric acid. The
elution profile was 8 to 66% acetonitrile in 15 min, followed by 66% acetonitrile isocratic for 8 min, from 66 to 95% acetonitrile in 0.5 min, and then 95% acetonitrile isocratic for 3 min. The flow rate was 0.7 ml/min. Detection occurred at a wavelength of 210 nm. In addition, aqueous samples were extracted with an equal volume of diethyl ether as described elsewhere (Bühler et al., 2000) followed by analysis of the ether phase by GC (see below) in order to determine substrate and product concentrations complementary to RP-HPLC analysis.

Samples from the organic phase of the two-liquid-phase biotransformations were diluted 200-fold in ice-cold diethyl ether containing 0.1 mM n-undecane as an internal standard and were dried over sodium sulfate. Toluene, benzyl alcohol, benzoic acid, and (R)-PAC were separated using a GC instrument (Fisons Instruments) equipped with an OPTIMA-5 fused silica capillary column (length, 25 m; inner diameter, 0.32 mm; film thickness, 0.25 μm) from Macherey-Nagel (Oensingen, Switzerland). The temperature profile was as follows: isotherm at 40°C for 2 min, from 40 to 100°C at 15°C/min, isotherm 100°C for 4 min, from 100 to 280°C at 50°C/min, and then 280°C isotherm for 5 min. Splitless injection with hydrogen as the carrier gas was used. Detection occurred by flame ionization. Identification and quantification of the substances were done by comparison with standards.

Results and discussion

Proliferation of plasmids pSPZ3 and pTEZ210 in E. coli. Agarose gel electrophoresis of plasmids extracted from an E. coli DH10B (pSPZ3, pTEZ210) culture confirmed stable proliferation and coexistence of both plasmids in E. coli cells (Fig. 5.2). This is possible due to the different incompatibility groups and selection markers of pTEZ210 (pSC101 replicon, Cm<sup>+</sup>) and pSPZ3 (pMB1 replicon, Km<sup>+</sup>). The size of plasmid pSPZ3 is nearly twice as large as the size of pTEZ210, which doubles also the intensity of the respective bands on the agarose gel. Nevertheless, a clearly higher copy number for pSPZ3 than for pTEZ210 can be estimated by comparing the intensities of the two plasmid bands in Fig. 5.2. Plasmid pSPZ3 is replicated in a “relaxed” fashion, which results in about 15–20 copies per cell whereas plasmid pTEZ210 is under stringent replicative control and thus is present only at five copies per cell at the maximum (Sambrook et al., 1989).

Formation of (R)-PAC from toluene in a multistep reaction catalyzed by recombinant E. coli. Resting E. coli DH10B (pSPZ3, pTEZ210) cells containing XMO and PDC<sub>W392M</sub> were tested as biocatalysts for the synthesis of (R)-PAC from 1.0 mM toluene as substrate on ml-scale (Fig. 5.3).
Toluene was rapidly oxidized to benzyl alcohol and benzaldehyde in a successive way by XMO. Benzaldehyde accumulated as the major intermediate product and served then as substrate for the formation of (R)-PAC by PDCW392M, for the formation of benzyl alcohol by host intrinsic dehydrogenases (see also Chapter 2), and for the further oxidation to benzoic acid by XMO (Bühler et al., 2000) (Fig. 5.3). The addition of pyruvate to a concentration of 2 mM did not have a significant effect on the biotransformation course (data not shown). Specific (R)-PAC formation rates of $7 \pm 1 \text{ mU/g CDW}$ and $8 \pm 0 \text{ mU/g CDW}$ were calculated based on the (R)-PAC concentrations determined after one hour of biotransformation in the absence and in the presence of pyruvate, respectively. An over-night biotransformation of 1.0 mM toluene yielded about 5 µM (R)-PAC as well as about 0.4–0.5 mM benzyl alcohol and benzoic acid without remaining benzaldehyde (Fig. 5.3), irrespective of the presence of 2 mM pyruvate (data not shown).

This experiment shows that (R)-PAC production from toluene by recombinant E. coli is possible, although productivities were relatively low.
The genotype of E. coli DH10B is optimized for the use of this strain in molecular biology, such as cloning of DNA that contains methylated cytosine or adenine residues, selection by α-complementation, the generation of genomic libraries, or plasmid rescue procedures (Gibco BRL, Gaithersburg MD). In contrast to E. coli DH10B, E. coli JM101 has been widely used as a host strain for biotransformations, also for the XMO-catalyzed multistep biotransformation of toluene (Bühler et al., 2000). In addition, the E. coli JM101 genome encodes the lacI gene sequence (genotype lacI'), whose product reduces the basal lac promoter-controlled gene expression level in the absence of inducer. Shaking flask cultivations resulted in higher maximal growth rates for E. coli JM101 than for E. coli DH10B under identical conditions (data not shown). The faster growth of E. coli JM101 points to a higher metabolic activity as compared to E. coli DH10B, which is desirable for cofactor dependent biotransformations. Therefore, E. coli JM101 was used as biocatalyst harboring plasmids pSPZ3 and pTEZ210 in further experiments.
The pyruvate decarboxylase-catalyzed formation of (R)-PAC from benzaldehyde. Benzaldehyde accumulated significantly faster than (R)-PAC in the biotransformation of toluene by E. coli DH10B (pSPZ3, pTEZ210) (Fig. 5.3). Resting cells of E. coli JM101 (pSPZ3) containing XMO were reported to oxidize toluene to benzyl alcohol and benzyl alcohol to benzaldehyde at specific rates of 100 and 95 U/g CDW, respectively (Bühler et al., 2000). Thus, benzaldehyde formation occurred about four orders of magnitude faster than the successive condensation to (R)-PAC. Apparently, the PDCW392Mcatalyzed reaction step is the bottleneck for the formation of (R)-PAC from toluene in this setup. Different E. coli biocatalysts were therefore tested for the formation of (R)-PAC from different concentrations of benzaldehyde as substrate (Fig. 5.4). The (R)-PAC formation rate of E. coli JM101 (pSPZ3, pTEZ210) was about four-fold higher with 30 mM than with 10 mM benzaldehyde (Fig. 5.4) and about 25-fold higher than in the biotransformation of 1.0 mM toluene using E. coli DH10B (pSPZ3, pTEZ210) as biocatalyst (Fig. 5.3). Significantly higher (R)-PAC formation rates were obtained for E. coli JM101 (pSPZ3, pTEZ210) when XMO gene expression was not induced (Fig. 5.4). This might be explained by a reduced metabolic burden due to the absence of XMO synthesis or of cell toxic effects provoked by n-octane used as an inducer. Small amounts of (R)-PAC were produced by bacteria of wild-type E. coli JM101 (Fig. 5.4), although the genome of this strain does not encode PDC gene sequences (Conway et al., 1987). Native acetohydroxyacid synthases (AHAS or acetolactate synthase) from E. coli strains are able to catalyze the production of (R)-PAC from benzaldehyde and pyruvate with a high enantiomeric excess (Engel et al., 2003; Engel et al., 2004). AHAS activity might explain the (R)-PAC formation in E. coli JM101 not overproducing PDCW392M.

The faster (R)-PAC formation at higher benzaldehyde concentrations is in accordance with earlier findings of enzyme kinetics. Isolated PDCW392M and AHAS operate with maximal efficiency at benzaldehyde concentrations in the range of 30–40 mM (Goetz et al., 2001; Engel et al., 2003). PDC from yeasts have a significantly higher affinity for benzaldehyde as compared to PDC from Z. mobilis and thus would represent an interesting alternative for this whole-cell application (Bringer-Meyer and Sahm, 1988).

E. coli KO20 contains the pet operon from Z. mobilis inserted into the pyruvate formate-lyase-encoding pfl gene. This engineered strain uses pyruvate for the production of ethanol catalyzed by wild-type PDC and alcohol dehydrogenase II from Z. mobilis rather than for the production of acetate and formate as the native pfl gene is inactivated (Ohta et al., 1991). E. coli KO20 produced (R)-PAC with lower specific activities as compared to the E. coli JM101 recombinants when incubated with 30 mM benzaldehyde (Fig. 5.4). Overexpression of
the pyruvate kinase gene encoded on plasmid pPYKec was reported to increase the intracellular pyruvate concentration from 0.5 ± 0.2 mM to 1.3 ± 0.4 mM (Emmerling et al., 1999). However, (R)-PAC formation was identical for wild-type E. coli KO20 and induced E. coli KO20 (pPYKec), indicating no significant effect of the intracellular pyruvate concentration on the PDCW392M activity (Fig. 5.4). The same results were obtained for induction of pyruvate kinase synthesis with a 20-fold lower IPTG concentration accordingly to (Emmerling et al., 1999) (data not shown).

**Fig. 5.4. PDC activity assays with resting cells of different E. coli strains.** Bacteria were incubated with 10 or 30 mM benzaldehyde as substrate. Substrate and product concentrations were determined after 60 minutes of biotransformation, which allowed the calculation of (R)-PAC formation rates. At least 30% of benzaldehyde was remaining at the end of biotransformations. Plasmids pSPZ3, pTEZ210, and pPYKec encoded the genes for XMO, PDCW392M, and pyruvate kinase, respectively. E. coli KO20 encoded the PDC gene on the genome (see “Materials and methods” for details). n. a., not available.
As a comparison, a suspension of *Z. mobilis* wild-type cells (24–26 g wet weight/liter) accumulated 2.8 mM (R)-PAC in 7.5 hours when incubated with an increasing concentration of benzaldehyde of 100 mM in total (Bringer-Meyer and Sahm, 1988). This corresponds to a specific activity of about 0.8 U/g CDW (assuming a water content of 70% of the wet cells (Madigan *et al.*., 2003)). Thus, the tested *E. coli* recombinants produced (R)-PAC with rates in the same order of magnitude as the *Z. mobilis* wild-type strain.

Benzaldehyde is relatively unstable in the *E. coli* host (Fig. 5.3). In resting *E. coli* JM101, benzaldehyde was reduced to benzyl alcohol with a specific rate of 15 U/g CDW presumably by cofactor dependent alcohol dehydrogenases (Bühler *et al.*, 2000). The degradation of benzaldehyde influences PDC catalyzed biotransformation in resting cells. Crude extracts prepared from the same strains that were used as biocatalysts in the previous experiment (Fig. 5.4) were tested for the formation of (R)-PAC with 30 mM benzaldehyde and pyruvate. Less than 0.5% of the benzaldehyde was reduced to benzyl alcohol in biotransformations with crude extracts, presumably due to the absence of cofactor regeneration (data not shown).

Similar to the whole-cell activity assays, (R)-PAC formation rates of *E. coli* JM101 (pSPZ3, pTEZ210) crude extracts were higher without induction of XMO synthesis (Fig. 5.5). Crude extracts from the *E. coli* JM101 wild-type bacteria showed also a residual (R)-PAC formation activity when incubated with benzaldehyde and pyruvate, and crude extracts from *E. coli* KO20 showed the same (R)-PAC formation rate irrespective of induction of pyruvate kinase synthesis. Specific initial (R)-PAC formation rates of *E. coli* KO20 crude extracts decreased faster with increasing biotransformation time as compared to the rates of the crude extracts of the *E. coli* JM101 recombinants. This can be explained by the higher stability of the mutated PDCW392M as compared to the wild-type PDC (Pohl, 1997; Goetz *et al.*, 2001). Initial specific activities of the *E. coli* KO20 extracts were higher than for the *E. coli* JM101 extracts, in spite of up to five fold more DNA templates per recombinant *E. coli* JM101 as compared to *E. coli* KO20 cell and a five-fold higher turnover number for PDCW392M than for the wild-type enzyme (Iding *et al.*, 1998). Thus, PDC synthesis seems to be more efficient in *E. coli* KO20 than in *E. coli* JM101 cells. However, direct determination of the PDC protein content in the crude extract was not possible by SDS-PAGE, as the PDC concentrations were too low to be quantified (not shown).

A possible reason for the relatively low PDCW392M gene expression and the absence of effects of the pyruvate kinase overproduction on (R)-PAC formation (Fig. 5.4 and Fig. 5.5) might be the reduced induction efficiency of the lac(-derived) promoters due to the use of glucose as the carbon source (Sambrook and Russell, 2001). However, preliminary
experiments with *E. coli* JM101 (pSPZ3, pTEZ210) grown on glycerol instead of glucose did not result in significantly higher specific \((R)\)-PAC formation rates (data not shown) and earlier studies also used glucose-containing medium for overproduction of pyruvate kinase in *E. coli* KO20 (pPYKec) (Emmerling et al., 1999).

Another reason might be a higher strength of the native *pfl* promoters, which control the PDC gene expression in *E. coli* KO20, as compared to the *lac* promoter, which controls the PDC\(^{W392M}\) gene expression on plasmid pTEZ210. Anaerobic growth conditions might further activate the *pfl* promoter controlled transcription and therefore might lead to higher PDC concentrations in *E. coli* KO20 (Reyes-Ramirez and Sawers, 2006). However, since oxygen is a prerequisite for XMO catalysis, the production of \((R)\)-PAC from toluene is precluded in anaerobically growing bacteria.

![Fig. 5.5. PDC activity assays with crude extracts of different *E. coli* strains.](image)

Crude extracts were incubated with 30 mM benzaldehyde and pyruvate. Concentrations of benzaldehyde and its metabolites were determined after 10, 20, and 30 minutes of biotransformation. \((R)\)-PAC formation rates were calculated based on \((R)\)-PAC concentrations, the biotransformation time, and on the total protein content of the crude extracts, determined by Bradford assays. Plasmids pSPZ3, pTEZ210, and pPYKec encoded the genes for XMO, PDC\(^{W392M}\), and pyruvate kinase, respectively. *E. coli* KO20 encoded the PDC gene on the genome (see “Materials and methods” for details).
In a control experiment, crude extract of *E. coli* JM101 (pPDC-W392M-His$_6$) overexpressing PDC$_{W392M}$ gene produced (R)-PAC at a rate of 61.0 ± 1.4 U/g prot$_{tot}$ (data not shown). Plasmid copy number of pPDC-W392M-His$_6$ is at least five fold higher than for pTEZ210, which might explain the 30-fold higher (R)-PAC formation rate when PDC$_{W392M}$ is overproduced in pPDC-W392M-His$_6$ than in pTEZ210 harboring *E. coli* bacteria. However, due to the same incompatibility groups of PDC-W392M-His$_6$ and pSPZ3, a stable proliferation of both plasmids in the same host cell is not possible. Nevertheless, this control shows the importance of a relatively high plasmid copy number to achieve higher intracellular PDC$_{W392M}$ concentrations, which results in higher specific (R)-PAC formation activities. These results suggest the construction of e.g. a single plasmid with higher copy number encoding the genes for both XMO and PDC in the same operon.

**Toxicity of reactants and biotransformations of toluene on liter scale.** Activity assays showed that (R)-PAC is most efficiently produced in the presence of relatively high benzaldehyde concentrations. Yet, in whole cells, benzaldehyde is efficiently reduced to benzyl alcohol by the enzymatic background activity. XMO catalyzed oxidation of benzyl alcohol to benzaldehyde can be used to counteract the dehydrogenase catalyzed benzaldehyde reduction, but XMO activity and pyruvate generation requires metabolically active bacteria. However, cell growth and metabolism might be affected by high concentrations of toxic reactants. The concentration dependent effects of toluene, benzyl alcohol, benzaldehyde, benzoic acid, and (R)-PAC on growth were therefore investigated for *E. coli* JM101. Benzaldehyde and toluene were inhibiting growth at significantly lower concentrations as compared to benzyl alcohol, benzoic acid, and (R)-PAC (Table 5.2).

Single phase biotransformations with *E. coli* JM101 (pSPZ3, pTEZ210) were performed in lab-scale bioreactors with the goal to produce and maintain relatively high benzaldehyde concentrations over a increased time period and to investigate the resulting effects on (R)-PAC production (Fig. 5.6). In a first biotransformation, toluene was added stepwise by pulses of 2 mM every 30 minutes in order to avoid the accumulation to critical concentrations (Fig. 5.6A and B). With this strategy, 21 of a total of 27 mmol toluene were oxidized by XMO. Only traces of toluene were detected in samples withdrawn during biotransformation. Toluene is relatively volatile, with a partition coefficient of 0.26 over air and water at 25°C (Amoore and Hautala, 1983), which makes the missing 22% of the fed toluene likely to be stripped out due to the aeration. In this biotransformation, benzaldehyde accumulated to a concentration of 1–2 mM, which was maintained for about four hours (Fig. 5.6A).
Table 5.2. Aqueous solubility and toxicity of toluene and derivatives for E. coli JM101

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximum aqueous solubility[^d] [mM]</th>
<th>Half-inhibiting concentration[^b] [mM]</th>
<th>Critical concentration[^c] [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>9.1</td>
<td>2 - 5</td>
<td>n. d</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>350</td>
<td>16</td>
<td>&lt; 32</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>72</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>(R)-PAC[^d]</td>
<td>n. a.</td>
<td>8 – 24</td>
<td>n. a.</td>
</tr>
</tbody>
</table>

[^a]: Solubilities at 30°C of toluene, benzyl alcohol and benzaldehyde, and benzoic acid were obtained from (Schwarz and Miller, 1980), (Barry et al., 1985), and (Das Gupta and Moulik, 1987), respectively.

[^b]: The concentrations at which the growth rate of E. coli JM101 is reduced to the half of the maximal growth rate.

[^c]: The minimal concentration at which growth of E. coli JM101 is completely inhibited.

[^d]: not available, since growth of E. coli JM101 was not completely inhibited by soluble benzoic acid concentrations.

However, benzaldehyde was constantly oxidized to benzoic acid by XMO and also efficiently reduced to benzyl alcohol by native alcohol dehydrogenases. The enzymatic background activity of E. coli JM101 is unable to oxidize benzaldehyde to benzoic acid (Bühler et al., 2000). These results thus indicate that both, XMO and alcohol dehydrogenases were still active when cell growth was completely inhibited about one hour after the first toluene addition. None of the reactants accumulated to concentrations that completely inhibited cell growth in toxicity assays (Table 5.2) but cumulative toxicity effects of all compounds present during biotransformation might have provoked the observed cell growth inhibition.

The presence of toluene in the cell broth was shown to kinetically inhibit the oxidation of benzaldehyde to benzoic acid by XMO in recombinant E. coli JM101 (Bühler et al., 2002). The benzaldehyde oxidation might therefore be minimized by an approach, which permanently provides toluene at small concentrations in the cell broth. In a second biotransformation, toluene was therefore fed continuously at a rate of 0.6 mM/min (Fig. 5.6C and D). Fully induced XMO converts toluene with a rate of about 100 U/g CDW (Bühler et al., 2000), which corresponds to a conversion of about 2 mM/min in this setup. Therefore XMO synthesis was reduced about four fold by induction with 0.002% instead of 0.02% (vol/vol) DCPK, as estimated from induction experiments with E. coli JM101 (pSPZ3) (Bühler et al., 2000). At this expression level, XMO activity is supposed to constantly convert most of the toluene fed whereas the continuous feed might reduce benzoic acid formation.
Coupling of metabolism and multistep biotransformation

B

- Sum of fed tolulene [mM]
- - Biomass [g CDW/liter]
- Acetate [g/liter]
- Glucose [g/liter]

D

- Sum of fed tolulene [mM]
- - Biomass [g CDW/liter]
- Acetate [g/liter]
- Glucose [g/liter]

F

- Sum of fed tolulene [mM]
- - Biomass [g CDW/liter]
- Acetate [g/liter]
- Glucose [g/liter]

Time [hours]

-3 -2 -1 0 1 2 3 4 5 6 7 8

-1 0 1 2 3 4 5 6 7 8 9

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8

-3 -2 -1 0 1 2 3 4 5 6 7 8
Fig. 5.6. Fed-batch based single-phase biotransformations of toluene by *E. coli* JM101 (pSPZ3, pTEZ210) on liter-scale. Induction of XMO and PDCW392M synthesis occurred 2–3 hours before addition of toluene as the substrate. Glucose served as the carbon source. Toluene addition represents time zero. (A, B) Step-wise addition of toluene with fully induced synthesis of XMO and PDCW392M. (C, D) Continuous toluene feed with fully induced PDCW392M but only partly induced XMO synthesis. (E, F) Continuous toluene feed with fully induced XMO and PDCW392M synthesis. (See “Materials and methods” for details).

This approach indeed resulted in a slower oxidation of benzaldehyde to benzoic acid and allowed the relatively rapid accumulation of 2 mM benzaldehyde in the first 20 min (Fig. 5.6C). However, benzaldehyde was reduced efficiently to benzyl alcohol by host-intrinsic dehydrogenases, which resulted in the accumulation of benzyl alcohol as the main product, probably as a drawback of the reduced XMO synthesis. Full induction of XMO synthesis seems to be necessary to efficiently counteract benzaldehyde reduction by native enzymes in the *E. coli* host. Although only traces of toluene were detected in the cell broth, the concentration was high enough to reduce benzoic acid formation. The content of lost toluene increased to 66% of the total of added toluene. Cell growth was inhibited about one hour after start of biotransformation (Fig. 5.6D).

In a third approach the same initial toluene feed rate was tested but XMO synthesis was fully induced by the addition of 0.02% (vol/vol) DCPK (Fig. 5.6E and F). In one hour, about 8 mM benzaldehyde was produced (Fig. 5.6E), which is a four fold higher concentration than achieved in the previous biotransformation (Fig. 5.6C). Subsequently, benzaldehyde was reduced to benzyl alcohol as the native dehydrogenase activity apparently was more enduring as compared to the XMO activity in the presence of cell growth inhibiting product concentrations. Cell lysis might have mainly affected the stability of the membrane bound XMO rather than of the soluble dehydrogenases. 61% of toluene was lost during biotransformation, which is slightly less than in the previous biotransformation and might be explained by the higher XMO activity.

(R)-PAC accumulated to less than 0.5 mM with maximal formation rates of 0.1–0.2 U/g CDW in all three biotransformations. The relatively high substrate and product concentrations significantly inhibited cell growth and also reduced XMO activity used for the formation of benzaldehyde from toluene and to counteract benzaldehyde reduction by the more stable native dehydrogenases. A prolongation of cell growth during biotransformation might therefore be useful for benzaldehyde production and stabilization, resulting in a faster and more enduring conversion to (R)-PAC.
Partition coefficients and two-liquid-phase biotransformations with bis(2-ethylhexyl)-phthalate and n-hexadecane as second phases. A second liquid phase that is present during biotransformation can extract toxic reactants from the cell-containing aqueous phase. An ideal setup might be useful for the production of high (aqueous) benzaldehyde concentrations without intoxicating the cells by toluene and benzyl alcohol. BEHP and n-hexadecane, two water immiscible and not cell-toxic organic solvents, were tested as second phases in this work. To evaluate the extractive potential, the partition coefficients of toluene and derivatives were determined for BEHP and n-hexadecane and compared to the coefficient with octanol as second phase (Table 5.3). Toluene was the most efficiently extracted solute in all tested two-phase systems. The relatively high partition coefficient allows the use of toluene as substrate up to molar concentrations in biotransformation setups with all three solvents tested as second phases without intoxication of the bacteria. Benzyl alcohol and benzaldehyde were extracted better by a second phase of BEHP and octanol than by a second phase of n-hexadecane, suggesting the highest benzaldehyde availability in a two-liquid-phase biotransformation setup with n-hexadecane. As a drawback, also benzyl alcohol has a relatively low partition coefficient, resulting in a 3–4 fold higher benzyl alcohol concentration in the aqueous than in the n-hexadecane phase. For a two-liquid-phase biotransformation with BEHP, n-hexadecane, or octanol as the second phases (phase ratio of 0.5), overall (R)-PAC concentrations of up to 29, 10, or 65 mM could be produced before growth of E. coli JM101 would be completely inhibited. The best extraction for benzyl alcohol and (R)-PAC would be obtained with a second phase of octanol (Table 5.3). However, the high toxicity of octanol for E. coli strains does not allow using this organic solvent as second liquid phase. Two-liquid-phase biotransformations with octanol or toluene as second phases might be possible by using solvent-tolerant P. putida strains as hosts (de Bont, 1998). A second toluene phase thereby would serve as substrate and at the same time extract the products.

The high standard deviation for the partition coefficient of toluene in a n-hexadecane-medium mixture and the significant difference to an earlier performed determination by (Abraham et al., 1994) reflects the difficulties to accurately determine toluene concentrations in the aqueous phase, presumably due to its high volatility (Table 5.3). Consequently, the partition coefficient of toluene in BEHP–medium might also be overestimated.
Table 5.3. Partitioning of toluene and derivatives in two-liquid-phase systems consisting of the aqueous phase and different organic solvent phases

<table>
<thead>
<tr>
<th>Solute</th>
<th>Partition coefficient for(^a)</th>
<th>Partition coefficient for(^b)</th>
<th>Partition coefficient for(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>2000 ± 360</td>
<td>19500 ± 10800</td>
<td>497 ± 57(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(527 ± 69)(^b)</td>
<td></td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>2.90 ± 0.66</td>
<td>0.294 ± 0.027(^c)</td>
<td>14.0 ± 2.1(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.303 ± 0.097)(^b)</td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>42.0 ± 6.7</td>
<td>12.3 ± 1.4(^c)</td>
<td>29.9 ± 0.5(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.6 ± 1.2)(^b)</td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>n. a.</td>
<td>1.52e-04 ± 1.3e-05</td>
<td>64.5 ± 13.6(^b)</td>
</tr>
<tr>
<td>((R))-PAC</td>
<td>2.60 ± 0.95</td>
<td>0.286 ± 0.014</td>
<td>7.08(^d)</td>
</tr>
</tbody>
</table>

aq., aqueous phase; n. a., not available.

\(^a\) Defined as the ratio of solute concentration in the organic to the concentration in the aqueous phase.

\(^b\) Data from (Abraham et al., 1994).

\(^c\) Determined in the presence of bacteria during biotransformation.

\(^d\) Estimated via “http://www.syrres.com/esc/est_kowdemo.htm”.

BEHP was used as the second phase for a two-liquid-phase biotransformation with *E. coli* JM101 (pSPZ3, pTEZ210) (Fig. 5.7A and B). Benzaldehyde accumulated as main product within five hours of biotransformation to an overall concentration of 47 mM. No \((R)\)-PAC was produced, although no significant benzaldehyde reduction to benzyl alcohol or oxidation to benzoic acid occurred in the first five hours. This might be explained by the low aqueous benzaldehyde concentration of maximally 1.3 mM as a result of the relatively high partition coefficient in this setup (Table 5.3). After biotransformation of about 23 hours, most benzaldehyde was reduced to benzyl alcohol. A significant content of the reactants was lost presumably due to stripping out via aeration. Cell growth was inhibited after 4–5 hours of biotransformation, which is reflected by an increase of the dissolved oxygen tension due to reduced respiration activity. However, the benzaldehyde concentration present in the cell broth did not significantly inhibit growth in toxicity assays performed in shaking flasks (Table 5.2). Presumably, bacteria were more sensitive to benzaldehyde during cultivation in the bioreactor due to the higher shear forces and the presence of a second liquid phase.

In a second two-liquid-phase biotransformation, \(n\)-hexadecane was used as the second liquid phase. In six hours, benzaldehyde accumulated to an overall concentration of 39 mM, which corresponded to 6.3 mM in the aqueous phase (Fig. 5.7C and D). In contrast to the biotransformation with BEHP as the second phase, the relatively high aqueous benzaldehyde
concentrations with a second phase of \( n \)-hexadecane allowed the formation of \( (R) \)-PAC. On the other hand, the high aqueous benzyl alcohol and benzaldehyde concentrations of 18.5 and 6.3 mM, respectively, inhibited cell growth already after one hour of biotransformation.

Interestingly, benzaldehyde was not reduced to benzyl alcohol in this setup as it was observed for the other biotransformations. The resulting availability of relatively high benzaldehyde concentrations allowed \( (R) \)-PAC formation over an increased time period of 20 hours. Benzaldehyde might have been stabilized by the use of glycerol instead of glucose as carbon source. Earlier studies on \( (R) \)-PAC production reported a decreased benzaldehyde reduction when yeast cells grew on pyruvate as compared to sucrose, probably due to a lower endogenous NADH level (Long and Ward, 1989). However, not only the dehydrogenase catalyzed benzaldehyde reduction but also the successive oxidation of toluene to benzyl alcohol and benzaldehyde by XMO are NADH-dependent, which makes a diminution of the endogenous cofactor pool not practical in this setup. Alternatively, the reduction of benzaldehyde by alcohol dehydrogenases might have been prevented by the high aqueous benzyl alcohol concentrations as a result of the relatively low partitioning to \( n \)-hexadecane used as second liquid phase (Table 5.3).

At comparable aqueous benzaldehyde concentrations of 6.3 and 8.7 mM, maximal \( (R) \)-PAC formation rates for the two-liquid-phase biotransformation with \( n \)-hexadecane was 0.03 U/g CDW (Fig. 5.7C) as compared to 0.19 U/g CDW for the single phase biotransformation (Fig. 5.6E), respectively. Although the intracellular pyruvate concentrations remain unclear, accumulation of acetate observed during all biotransformations indicates the availability of pyruvate, given that acetate was generated from pyruvate. Acetate accumulation takes place as a part of a stress response to incubation with toxic chemicals, due to an overflow metabolism, or by the decarboxylase activity of PDCW392M via the formation of acetaldehyde. A lower pyruvate availability due to a reduced overflow metabolism for the cells of the two-liquid-phase biotransformation grown on glycerol as compared to the cells of the single phase biotransformation grown on glucose thus might explain the lower \( (R) \)-PAC formation.
Fig. 5.7. Two-liquid-phase fed-batch based biotransformations of toluene by *E. coli* JM101 (pSPZ3, pTEZ210) on liter-scale. (A, B) Biotransformation with BEHP used as second phase (phase ratio of 0.5; two liter working volume). Induction of XMO took place with the addition of the second phase, whereas PDCW392M was already induced during overnight batch cultivation. Addition of 100 mmol toluene as substrate (represents time zero) occurred one hour after addition of the second phase. During this biotransformation, no *(R)*-PAC was detected, and its concentration therefore is not indicated in the figure. (C, D) Biotransformation with *n*-hexadecane as second phase (phase ratio of 0.27; 1.1 liter working volume). Induction of XMO and PDCW392M occurred three hours before addition of the second phase, which contained 240 mmol toluene as substrate. (See “Materials and methods” for details).
PDCW392M has a relatively low affinity not only for benzaldehyde but also for pyruvate as cosubstrate for the carboligation reaction. The higher stability of PDCW392M as compared to the wild-type PDC due the tryptophan substitution coincides with a 3–7 fold increase of the Michaelis-Menten constant (K_m) for pyruvate (Pohl, 1997). In this study, the use of E. coli overproducing pyruvate kinase or the addition of 2 mM pyruvate to the whole-cell biotransformation did not have a significant effect on (R)-PAC formation rate. Nevertheless, the use of a metabolically engineered host strain, such as E. coli YYC202, which is completely blocked in its ability to convert pyruvate into lactate, acetyl-CoA, or acetate, might further accelerate (R)-PAC formation by accumulating up to 62 g/liter pyruvate (Zelic et al., 2003).

Conclusions and outlook

We showed the feasibility of (R)-PAC production from toluene by a novel route in this study. Although productivities are not competing with current industrial processes, our results provide a starting point for further optimization work. (R)-PAC production might be significantly increased by more efficiently overproducing a PDC variant with an increased substrate affinity in a pyruvate accumulating host strain.

PDC is able to decarboxylate pyruvate and successively condense it with benzaldehyde to (R)-PAC. This reaction is interesting, on the one hand due to the synthesis of a value-added product from relatively cheap substrates. On the other hand, pyruvate used as a cosubstrate for PDC is a key metabolic intermediate in the cell. Thus (R)-PAC production couples the activity of the central carbon metabolism with a heterologous enzyme activity. Here, mass spectrometrically based flux analysis of biocatalysts that use 13C-labeled glucose as energy source would allow investigating the channeling of pyruvate to the synthesis of (R)-PAC, metabolites, or amino acids under different biotransformation conditions.

Acknowledgements

We are indebted to Michael Breuer for providing plasmid pPDC-W392M-His6 and (R)-PAC as an analytical standard, to Uwe Sauer for providing E. coli KO20 and plasmid pPYKec, to Patty Kleinsteuber and Anja Kulmsee for excellent technical assistance. This work was supported by grants from the European Commission (QLK3-2001-00435, acronym BARTOLO).
Chapter 6: Concluding remarks and outlook

Daniel Meyer
Recombinant bacteria represent an interesting and valuable tool for the production of chemicals in applied biocatalysis. In order to be useful on a versatile level, this tool has to work reliable and efficient and must not change its catalytic performance in time.

However, change is associated with evolution, which represents the base for life, also for bacteria used as biocatalysts. Overcoming this natural biocatalyst instability by the generation of “evolution-free” host bacteria, thus, might represent a long term goal of applied biocatalysis.

Evaluation and understanding of the physiology of an individual bacterium rather than of the whole culture or colony is thereby the first step for the development of inherently stable biocatalysts. Today, gene expression dynamics can be investigated on the single cell level (Guido et al., 2006; Mettetal et al., 2006). This technology might also be useful to better understand the reasons for the high variation of gene expression levels observed in solvent-tolerant host bacteria. Current developments in micro-separation techniques might soon allow profiling the genome, the transcriptome, the proteome, and the metabolome of a single bacterium in a routine way (Liu et al., 2006). Here, the PDC catalyzed condensation of benzaldehyde and endogenous pyruvate would be a useful model reaction for metabolic flux analysis of a bacterium, not only while growing under physiological conditions but also during biotransformations.

Stress promotes the generation of errors. For example, the overexpression of heterologous genes at high expression rates drains resources of the host, which ultimately can lead to the synthesis of inactive enzymes e.g. due to the incorporation of wrong amino acids during elongation or incorrect protein folding. In addition, bacteria are stressed when they are exposed to unnatural conditions during biotransformation, including high shear forces due to vigorous stirring or high concentrations of toxic substrates or products. Bacteria “sense” such environmental changes via their membrane envelope. The membrane composition therefore is supposed to provide information about the physiological state of a biocatalyst. In this study, we showed that the complete lipid composition can be identified by state-of-the-art chromatography, which exceeds the potential of traditional fatty acid analysis and may contribute significantly to the emerging field of lipidomics (Wenk, 2005).

Metabolic engineering might be useful to generate “stress-proof” hosts. For example, increasing energy generation by accelerating the central metabolism of the host and channeling the energy to the protein synthesizing machinery and cofactor regeneration might result in more efficient biocatalysts with a more stable catalytic performance.
Bacterial solvent tolerance is interesting for the design of new two-liquid-phase biotransformation setups. However, soil bacteria, such as solvent-tolerant *P. putida* strains are also known as effective degraders of many aromatic chemicals. Such bacteria have often a rather complex catabolism, which enables them to use a broad range of nutrients in order to avoid starvation phases. This complexity is not useful for biocatalysts growing on a defined medium. We showed that the catabolic activity of the host strain can drastically reduce the yield of a biotransformation. Recalcitrant xenobiotics, such as nitroaromatic, were efficiently degraded by *E. coli* cells, which are primary inhabitants of the intestines of warm-blooded animals and thus are not supposed to have developed degradation activities for a wide number of aromatic chemicals. Our results indicate that the catabolic potential of host bacteria cannot always be deduced from their natural primary habitat or from their preferred “lifestyle”. The deletion of product degrading enzyme activities by means of mutation can be used to make the enzymatic background activity compatible to the desired biotransformation. A bottom-up approach would be the generation of host strains that contain only functions relevant for biocatalysis. The minimum requirements for a host strain include efficient synthesis of heterologous enzymes, cofactor regeneration, and providing a stable environment for the reaction to be catalyzed. A minimal gene set to sustain a functioning cell under ideal conditions is supposed to consist of about 250 genes, which is 17-fold less than the total number of *E. coli* genes (Koonin, 2000). The reduced complexity of such a host strain might cause a higher efficiency and reproducibility of biotransformations and the missing degradation pathways might allow the production of a broader range of chemicals.

This thesis highlights the important role of the host physiology for the efficiency of oxidative biotransformations. Host strain selection might become an equally important issue for the design of future bioprocesses as e.g. the optimization of cultivation conditions, selection of an expression system, or the use of appropriate product separation technologies. The current advent of new technologies for analyzing and engineering the physiological and metabolic state of host bacteria during biotransformation will contribute to the development towards the “ideal” host.
Literature


Literature


Curriculum vitae
**Personal data**

Name: Daniel Philippe Meyer  
Born: November 29, 1975 in Sursee, Switzerland  
Nationality: Swiss  
Family status: Single

**Education**

1982–1988  
Primarschule Schenkon, Switzerland

1988–1995  
Kantonsschule Sursee, Switzerland  
*Graduation: Matura typus C*

1995  
Swiss military service

1996–1998  
Molecular- and microbiology studies (Biologie II)  
University of Basel, Switzerland  
*Graduation: 1. & 2. Vordiplom*

1998–2001  
Biotechnology studies  
Ecole Supérieure de Biotechnologie de Strasbourg (ESBS), France  
European School of the Association of the Upper Rhine Universities (EUCOR)  
*Graduation: Diplôme d’ingénieur en biotechnologie (Dipl. Biotech.)  
Diplôme des études approfondies (DEA) de biologie moléculaire et cellulaire (option biotechnologie)*

2002–2006  
Ph.D. studies supervised by Prof. Dr. Bernard Witholt, Institute of Biotechnology, ETH Zürich, Switzerland and Prof. Dr. Andreas Schmid, Chair of Chemical Biotechnology, University of Dortmund, Germany
Publications

Neumann, G., Kabelitz, N., Zehnsdorf, A., Miltner, A., Lippold, H., Meyer, D.,
Pseudomonas putida DOT-T1E to a second phase of a solvent for economically

and Pseudomonas putida strains for selective biotransformation of m-nitrotoluene


Research presentations

Oral presentations

catalyst for selective hydroxylation of aromatic and heteroaromatic compounds?
Annual Conference of the Association for General and Applied Microbiology

Poster presentations

m-nitrotoluene. 11th European Congress on Biotechnology, ECB11. August 24-29.
Basel, Switzerland.

solvent-efflux pumps on preparative biooxidations. 5th D-BIOL Symposium of ETH

A search for the optimal recombinant host for specific nitrotoluene oxidation.