Multi-enzymatic routes for the targeted synthesis of enantiopure vicinal amino alcohols

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Chapter 2
A Chimeric Styrene Monooxygenase with Increased Efficiency in Asymmetric Biocatalytic Epoxidation

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Abstract. The styrene monooxygenase (SMO) system from Pseudomonas sp. is constituted by the two enzymes StyA and StyB. StyB catalyzes the reduction of FAD at the expense of NADH. After the transfer of FADH₂ from StyB to StyA, reaction with O₂ generates FAD-OOH that is the epoxidizing agent. The waste of redox equivalents due to a partial diffusive transfer of FADH₂, the insolubility of recombinant StyB and the impossibility to express StyA and StyB in a 1:1 molar ratio reduce the catalytic efficiency of the natural system. Herein, we present a chimeric SMO (Fus-SMO) that was obtained by genetic fusion of StyA and StyB with a flexible linker. E. coli cells expressing Fus-SMO possess ca. 50% higher activity for the epoxidation of styrene derivatives than E. coli cells expressing separated StyA and StyB as a combination of: i) balanced and improved expression levels of reductase and epoxidase units; ii) intrinsic higher specific epoxidation activity of Fus-SMO in some cases. The epoxidation activity of purified Fus-SMO was up to 3-fold higher than two-component StyA-StyB (1:1, molar ratio) and up to 110-fold higher than natural fused SMO. Determination of coupling efficiency and study on the influence of O₂ pressure were also performed. Finally, Fus-SMO and formate dehydrogenase were coexpressed in E. coli and applied as self-sufficient biocatalytic system for the epoxidation above 500 mg scale.
2.1 Introduction

Chiral epoxides are important building blocks in organic synthesis because of their high versatility and reactivity towards a variety of reagents. Indeed, they can undergo $S_N2$ reactions with a large variety of nucleophiles. Furthermore, epoxides find large applications as intermediates for the synthesis of active pharmaceutical ingredients, natural products, flavors and fragrances, other fine chemicals and advanced polymeric materials.\(^1\)-\(^{22}\) Moreover, epoxide moieties are also found in naturally occurring APIs, such as epothilones,\(^{23}\) a class of potential cancer drugs (Figure 2.1).

Chiral epoxides are classically synthesized via asymmetric epoxidation of alkenes using $\text{Ti(O-iPr)}_4$ (i.e. Katsuki-Sharpless)\(^{24}\) or salen-Mn(III) complexes (i.e. Jacobsen),\(^{25}\) which require stoichiometric amounts of a chemical oxidant. Hydrolytic kinetic resolution of racemic epoxides is also possible using chemical methods\(^2\),\(^8\),\(^{26}\) as well as biocatalytic methods involving hydrolases.\(^3\),\(^{27-31}\) Other more recent methodologies involve iron-based catalysts with hydrogen peroxide\(^{32-34}\) or organocatalysts with an oxidant such as hydrogen peroxide, oxone, hypochlorite salts, peroxides (e.g. TBHP, mCPBA) or trichloroisocyanuric acid, etc.\(^{35-40}\) Despite of significant research efforts and progresses in the field of chemocatalytic and organocatalytic asymmetric epoxidation of terminal alkenes such as styrene and derivatives, achieving elevated stereoselectivity (99% ee or higher) remains still a
challenge.\textsuperscript{35,36,38,41-51} Hence, the biocatalytic counterpart of this reaction has been investigated during the past fifteen years using either flavin (FAD) or iron-dependent monooxygenases.\textsuperscript{52} Enzymatic epoxidation is particularly attractive as epoxides are usually obtained with elevated enantiomeric excess (>99%) using molecular oxygen as oxidant. Among the others, the bi-enzymatic system of the FAD-dependent styrene monooxygenase (SMO) from \textit{Pseudomonas} sp. has been exploited for the production of enantiopure styrene oxide (and derivatives thereof) in laboratory and pilot-scale production employing fermenting or resting recombinant \textit{E. coli} cells\textsuperscript{53-56} as well as crude enzyme preparations.\textsuperscript{57} A thorough comparison between the SMO enzymatic process and different chemical epoxidation processes has shown that the former is the most advantageous when economic profitability and environmental impact are concomitantly considered.\textsuperscript{58} The potential of SMOs in chemical synthesis has also been demonstrated in the production of chiral vicinal diols, amino-alcohols, $\alpha$-hydroxy-carboxylic acids and $\alpha$-aminoacids through one-pot, concurrent multi-step cascades.\textsuperscript{59-62} The current drawback regarding the use of the natural SMO enzymatic system, as in \textit{Pseudomonas} sp., is the requirement of two separated enzymes (StyA and StyB) in order to promote the efficient epoxidation activity.\textsuperscript{63} Hence, both enzymes are usually coexpressed in \textit{E. coli}.\textsuperscript{53} StyB catalyzes the reduction of FAD to FADH$_2$ at the expense of NADH, whereas StyA utilizes FADH$_2$ and O$_2$ to generate FAD-OOH in its active site. The final epoxidation of the styrene substrate, performed by StyA, regenerates the oxidized FAD upon dehydration (Figure 2.2). The actual mechanism of this bi-enzymatic process is still matter of debate. Early studies have reported a pure diffusive transfer of FADH$_2$ from StyB to StyA.\textsuperscript{64} Nonetheless, subsequent in-depth studies strongly support the existence of a molecular interaction during the catalytic cycle between StyB and StyA from \textit{Pseudomonas} sp. as well as other SMOs.\textsuperscript{65-67} Published kinetic data show: \textit{i}) the existence of two competitive mechanisms (diffusive and channeling) for the transfer of FADH$_2$ from StyB to StyA;\textsuperscript{65-67} \textit{ii}) a variation of the epoxidation activity of StyA in presence of different types of StyB, wherein the highest rate was observed in combination with the natural partner.\textsuperscript{67}
Figure 2.2. Simplified catalytic cycle of the flavin-dependent styrene monooxygenase (SMO). a) Step 1: Reduction of FAD to FADH$_2$ catalyzed by StyB and further transfer to StyA, whereby oxidation to FAD-OOH occurs. b) Step 2: Asymmetric epoxidation of styrene (or derivatives) by StyA with regeneration of oxidized FAD.

Whilst naturally occurring fused SMOs (StyA2B) have been isolated, the catalytic activity was from one to two orders of magnitude lower than the bi-enzymatic SMO system from *Pseudomonas* sp.\textsuperscript{68} Interestingly, the epoxidation activity of StyA2B increased when an additional epoxidase enzyme (StyA1) was included.\textsuperscript{67} All these findings reveal that the molecular interaction between the different enzymatic units has important synergistic effects on the overall catalytic cycle, besides a mere improved transfer of FADH$_2$ from one unit to the other one. However, StyA is capable of catalyzing the epoxidation also in absence of StyB as long as reduced FAD is supplied. This property was exploited for the generation of hybrid chemo-enzymatic and electro-enzymatic systems.\textsuperscript{69-71} So far, the catalytic efficiency of these “StyA-hybrid” systems was significantly lower than that of the natural bi-enzymatic StyA-StyB. This reduced efficiency may be, in part, attributed to the lack of catalytic activation on StyA effected by StyB. Additionally, it has been shown that: i) the highest epoxidation activity is obtained when StyA and StyB are combined at ca. 1:1 ratio and at low FAD concentration (ca. 15 µM)\textsuperscript{64,65,67} and ii) the reduction of oxidized FAD by StyB is the rate-limiting step.\textsuperscript{66,72} Obtaining a nearly 1:1 ratio mixture of recombinant StyA and StyB in *E. coli* and in active form is still a challenging task. One issue is the difficult balancing and regulation of the expression
of both genes. The second, more severe, issue is that recombinant StyB in E. coli is mainly obtained as insoluble inclusion bodies (i.e. denatured form). In fact, in vitro experiments always required refolding of the inactive StyB, a lengthy and low-yielding procedure.\textsuperscript{64-66}

Herein, we present a chimeric SMO in which reductive (StyB) and epoxidation (StyA) enzymatic units are fused with a flexible linker of 30 amino acids\textsuperscript{73} in order to: i) solve the insolubility issue of StyB, ii) maximize the epoxidation activity (StyA–StyB 1:1 ratio), iii) improve FADH\textsubscript{2} transfer and find an optimum balance with coupling efficiency (NADH consumption vs. styrene epoxidation). A recent study reported on the catalytic mechanism of other artificially fused SMOs.\textsuperscript{72} Nevertheless, these chimeric enzymes were either produced in insoluble (i.e. non active) form or possessed lower catalytic rate for the epoxidation of styrene (ca. 5-fold or less) compared to the natural system. The reason for this discrepancy compared to the present work will also be discussed.

2.2 Results and Discussion
2.2.1 Design of the fused SMO and initial tests for activity
The StyA and StyB genes belonging to the bi-enzymatic system of the styrene monooxygenase (SMO) from \textit{Pseudomonas sp.} were genetically fused using a flexible linker\textsuperscript{73} which was constituted by 30 amino acids (\textit{section 2.4.3}). The gene construct (Fus-SMO) was designed in the following order: (N-His\textsubscript{6}-tag)-(StyA)-(linker)-(StyB). Positioning the His\textsubscript{6}-tag DNA sequence downstream from the T7 promotor and upstream from the StyA gene confers normally enhanced level of enzyme expression. The gene of the StyB was positioned at the end of the construct as this enzyme alone has been always expressed mainly as inclusion bodies (>95% of inactive enzyme).\textsuperscript{65} \textit{E. coli} BL21 D3 cells were transformed with the DNA encoding for the chimeric Fus-SMO enzyme and cells were grown on agar plate. Due to the different morphologies of the \textit{E. coli} colonies obtained (i.e. various colors: pink, blue, white), four of them were selected for further testing of expression, solubility and activity (\textbf{Figure 2.3}). The generation of pigmented cells stems from the production of indigo (i.e. blue color) or indirubin (i.e. red) during cultivation, which is enabled by the overexpressed Fus-SMO.\textsuperscript{74-76}
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In preliminary experiments, the conversion for the epoxidation of styrene (1a) was determined after a specific reaction time, using lyophilized E. coli whole cells overexpressing Fus-SMO in a biphasic system (aqueous buffer/n-decane, (Table 2.1). As reported in literature,54,60 the organic phase acts as a reservoir of styrene and reduces the molecular toxicity of the product styrene oxide. Furthermore, it prevents the spontaneous opening of the epoxide ring. Interestingly, the quantity and solubility of the expressed Fus-SMO into the cells seems to not correlate with a particular pigmentation of the host organism (Figure 2.3), whereas a difference

Figure 2.3. SDS-PAGE for the expression of Fus-SMO in E. coli BL21 DE3 as host (top); SDS-PAGE for verifying the soluble part of the expressed Fus-SMO when expression was performed at 25 °C overnight (bottom). Marker: PageRuler™ Unstained Protein Ladder (ThermoFisher Scientific)
in the conversion was observed only in one case for colony 2 (Table 2.1). However, further optimization of the expression conditions (i.e. IPTG concentration) revealed that all the *E. coli* colonies perform the epoxidation equally well, independently from their pigmentation.

**Table 2.1.** Conversions [%] of 1a (20 mM) to (S)-2a for the activity test of lyophilized whole cells containing Fus-SMO with and without the addition of FAD (50 µM).

| Entry | colony | conversion of 1a after expression of Fus-SMO at 37 °C for 3 h [%] | - FAD | + FAD |
|-------|--------|---------------------------------------------------------------|-------|-------|
| 1     | 1      | 24                                                           |       | 24    |
| 2     | 2      | 7                                                            |       | 24    |
| 3     | 3      | 20                                                           |       | 56    |
| 4     | 4      | 21                                                           |       | 50    |

| Entry | colony | conversion of 1a after expression of Fus-SMO at 30 °C for 5 h [%] | - FAD | + FAD |
|-------|--------|---------------------------------------------------------------|-------|-------|
| 5     | 1      | 40                                                           |       | 22    |
| 6     | 2      | 39                                                           |       | 26    |
| 7     | 3      | 40                                                           |       | 26    |
| 8     | 4      | 38                                                           |       | 23    |

| Entry | colony | conversion of 1a after expression of Fus-SMO at 25 °C o.n. [%] | - FAD | + FAD |
|-------|--------|---------------------------------------------------------------|-------|-------|
| 9     | 1      | 55                                                           |       | 52    |
| 10    | 2      | 74                                                           |       | 99    |
| 11    | 3      | 53                                                           |       | 52    |
| 12    | 4      | 52                                                           |       | 48    |

The influence of the addition of exogenous FAD was also assayed (50 µM) during the reaction. Biocatalytic reactions in presence or absence of FAD afforded statistically analogous results. Hence, *E. coli* is capable of producing sufficient amount of cofactor in combination of Fus-SMO to sustain the reaction (Table 2.1). Nevertheless, for the sake of reproducibility of the results, this whole study was carried out by adding a minimal amount of FAD to each biocatalytic reaction because long time storage of *E. coli*/Fus-SMO (frozen pellets or lyophilized cells) was possible, but data on the stability of FAD in such conditions were not available. A glycerol stock solution prepared from the culture obtained from colony 1 was
used for the continuation of this study. Optimization of the expression conditions (25 °C, 16 h, IPTG 0.1 mM) and further testing of activity led to the production of soluble and active Fus-SMO in elevated amount (Figure 2.4).

As shown in Table 2.2, whole lyophilized E. coli cells carrying the overexpressed Fus-SMO, under different conditions, revealed good conversions in all tested cases. All the further studies were carried out by inducing enzyme expression with the minimum amount of IPTG (0.1 mM) since no significant discrepancy was observed.

| Entry | lyophilized cells [mg mL⁻¹] | 0.1 mM IPTG 2a [%]ᵦ | 0.5 mM IPTG 2a [%]ᵦ | 1 mM IPTG 2a [%]ᵦ |
|-------|-----------------------------|----------------------|----------------------|-------------------|
| 1     | 5                           | 81±7                 | 69±19                | 55±9              |
| 2     | 10                          | >99                  | 85±2                 | >99               |
| 3     | 20                          | >99                  | 95±6                 | >99               |

Reactions were performed in duplicates; the reported conversion is the average of two measurements.

Moreover, as the biocatalytic epoxidation may also be influenced by the availability of dioxygen in the headspace, different reaction vessels were already considered (Table 2.3). Quantitative epoxidation of 1a (10 mM) in 1 mL of biphasic reaction mixture (aqueous buffer/n-decane, 1:1, v:v⁻¹) was obtained using 4 mL glass vials as
reaction vessel. Employing vials with smaller volume (2 mL) led to a maximum of 41% conversion, likely due to insufficient availability of dioxygen. The use of vials with larger volume (e.g. 20 mL) is possible, although agitation must be carefully set in order to assure an efficient mixing of the biphasic mixture.

Table 2.3. Bio-conversion [%] of 1a (10 mM) to (S)-2a by the soluble fraction of Fus-SMO expressed with different IPTG concentrations using a variation of reaction vessels.

| Entry | IPTG [mM] | 2 mL Eppendorf tubes 2a [%] | 4 mL glass vials 2a [%] | 20 mL glass vials 2a [%] |
|-------|-----------|-----------------------------|-------------------------|-------------------------|
| 1     | 0.1       | 40                          | >99                     | 50                      |
| 2     | 0.5       | 34                          | >99                     | 55                      |
| 3     | 1         | 41                          | >99                     | 54                      |

The bi-enzymatic StyA-StyB system has been often applied in a biphasic system, in which the organic phase was a high boiling solvent such as hexadecane, bis-(2-ethylhexyl)phthalate, etc. In preliminary experiments for the reaction in preparative scale, the difficult final evaporation of the high boiling decane led to a troublesome and high energy-consuming work-up procedure. More experiments also showed that it is possible to use low boiling organic solvents, such as n-heptane or even neat styrene, without affecting the productivity if lyophilised cells are employed (Table 2.4). Hence, a mixture of aqueous buffer (KPi, pH 8.0, 50 mM) and n-heptane (1:1 v v⁻¹) was selected for further experiments.
Table 2.4. Bio-epoxidation of 1a (20 mM) by Fus-SMO (20 mg mL⁻¹) in different solvent systems.

| Entry | KPi buffer [µL] | Cells [mg] | Subs. [µmol] | Organic solvent [µL] (v/v) | Conv. (S)-2a [%][a] | (S)-2a formed [µmol] | Productivity [µmol (S)-2a/mg cells] |
|-------|----------------|------------|--------------|-----------------------------|---------------------|----------------------|-----------------------------------|
| 1     | 500            | 10         | 10           | hexane [500](50%)           | >99                 | >9.9                 | >0.99                             |
| 2     | 1000           | 20         | 20           | -                           | 67±5                | 13.3                 | >0.67                             |
| 3     | 950            | 20         | 435          | styrene [50](5%)            | 5±0                 | 19.6                 | 0.98                              |
| 4     | 980            | 20         | 20           | decane [20](2%)             | 68±2                | 13.6                 | 0.68                              |
| 5     | 960            | 20         | 20           | decane [40](4%)             | 69±3                | 13.8                 | 0.69                              |
| 6     | 940            | 20         | 20           | decane [60](6%)             | 69±1                | 13.6                 | 0.68                              |
| 7     | 920            | 20         | 20           | decane [80](8%)             | 70±2                | 14.0                 | 0.70                              |
| 8     | 900            | 20         | 20           | decane [100](10%)           | 72±3                | 14.4                 | 0.72                              |
| 9     | 500            | 10         | 10           | decane [500](50%)           | >99                 | >9.9                 | >0.99                             |
| 10    | 500            | 10         | 10           | heptane [500](50%)          | >99                 | >9.9                 | >0.99                             |

[a] Reactions were performed in duplicates and the reported conversion is the average of two measurements. The optical purity was measured for selected samples (one per each condition) by chiral HPLC. In all cases, the enantiomeric excess was >99% (S).

2.2.2 Determination of the coupling efficiency of Fus-SMO

The coupling efficiency of the StyA-StyB system (bi-enzymatic or fused) is defined as the ratio between the quantity of substrate epoxidated and the reducing equivalents consumed. Detailed biochemical studies have revealed that the coupling efficiency is a function of: i) the relative concentration of StyA and StyB, ii) the substrate concentration and iii) the FAD concentration. A coupling efficiency verging towards one was measured at low FAD concentration (1 µM or less) and a StyA/StyB ratio of ca. 500. In this condition, StyB produces FADH₂ in extremely low concentration (nM range) and it can be quantitatively transferred to StyA. Hence, StyA produces FAD-OOH that can be almost quantitatively consumed for the epoxidation of styrene. Therefore, virtually no FADH₂ is wasted in the generation of H₂O₂ as by-product. The logic drawback of this specific reaction condition is that the epoxidation activity is dramatically reduced, making it inapplicable for synthetic
purposes. Therefore, the challenge is to maximize the coupling efficiency without affecting the overall epoxidation activity. We hypothesized that our Fus-SMO may have improved coupling efficiency at reaction conditions that are suitable for a high epoxidation rate. In the synthetic set-up reported in this work, NAD⁺ was applied in catalytic amount (1 mM) and recycled by a formate dehydrogenase from Candida boidinii (Cb-FDH) and HCOONa. A set of experiments were performed in which the equivalents of HCOONa (i.e. the ultimate source of reducing equivalents) were gradually increased from 0 to 5. As depicted in Figure 2.5, only four equivalents of formate were required in order to reach full conversion of 1a (20 mM). However, 20% conversion of 1a was observed even without any addition of HCOONa. The hypothesis is that some endogenous enzymes from the E. coli lyophilized cells (10 mg mL⁻¹) can somehow regenerate, in part, the NADH cofactor. Taking into account this background activity, the remaining 80% conversion of 1a was driven by only 4 equivalents of HCOONa corresponding to a remarkable estimated coupling efficiency of ca. 20%. The product (S)-2a was obtained in enantiopure form (ee >99% S).

**Figure 2.5.** Conversion of 1a (20 mM) to (S)-2a at varied concentration of HCOONa as final hydride donor. The bio-transformations were performed in a biphasic system KPi (pH 8.0, 50 mM)/n-heptane (1:1 v/v, 1 mL total reaction volume) containing E. coli/Fus-SMO lyophilized cells (10 mg mL⁻¹), NAD⁺ (1mM), FAD (50 µM), HCOONa (0-100 mM) and Cb-FDH (10 µM). The mixtures were incubated at 30 °C, 180 rpm for 24 h. Conversions are the average of two independent sets of experiments, both in duplicate (for all dataset including standard deviations see section 2.4.6). The enantiomeric excess was determined by chiral HPLC to be >99% (S).
2.2.3 Influence of the dioxygen pressure

Preliminary investigation has shown that the availability of dissolved molecular oxygen might become the limiting factor for the biocatalytic epoxidation by Fus-SMO under particular reaction conditions (data not shown). It has been reported that the activity of some oxygenases can be strongly enhanced by using pure O₂ in the headspace at atmospheric pressure or even under pressure.⁷⁷,⁷⁸ In order to assess properly the biocatalytic performance of our Fus-SMO, we carried out a comparative study with the original bi-enzymatic StyA-StyB construct (pSPZ10) by Panke and coworkers.⁵³ The pSPZ10 plasmid containing the styA and styB genes encoding for the SMO bi-enzymatic system of Pseudomonas sp. strain VLB120 was kindly donated by Prof. Sven Panke.⁵³,⁶³,⁷⁹

Expression of StyA-StyB with pSPZ10 plasmid was initially performed in E. coli JM101 according to literature. The expression in E. coli Arctic Express cells was also tested, as this strain is capable of improving expression of insoluble proteins such as StyB and this strain was not commercially available at the time of Panke’s study. Although expression of soluble StyA was significantly superior in E. coli JM101 (Figure 2.6), the tests of conversion vs time with lyophilized cells of E. coli JM101 and E. coli Arctic Express provided similar results (Table 2.5). This observation corroborates the assumption that the inefficient expression of soluble StyB in the bi-enzymatic system is the limiting factor.

Figure 2.6. SDS-PAGE for the expression of pSPZ10-StyAStyB in E. coli JM101 and Arctic Express cells. The SDS-PAGE was visualized using a gel imaging system from Biorad which does not visualize proteins lacking of tryptophan residues (chaperonin from Arctic Express cells). Marker: PageRuler™ Unstained Protein Ladder (ThermoFisher Scientific); MW_{StyA} = 46 kDa and MW_{StyB} = 18 kDa
Table 2.5. Results for the bio-catalytic conversion of 1a (10 mM) to (S)-2a for the comparison of *E. coli* JM101 and Arctic Express cells as host organism for the expression of pSPZ10-StyAStyB.

| entry | cells                                   | JM101 2a [%][a] | Arctic Express 2a [%][a] |
|-------|-----------------------------------------|-----------------|-------------------------|
| 1     | wet fresh cells (180 mg mL⁻¹)            | 37±1            | 72±13                   |
| 2     | frozen pellet (130 mg mL⁻¹)              | 78±<1           | 95±<1                   |
| 3     | lyophilized whole cells (20 mg mL⁻¹)     | 90±2            | 95±<1                   |

[a]Reactions were performed in duplicates and the reported conversion is the average of two measurements.

Finally, the rates of the epoxidation reaction using *E. coli* BL21 DE3/Fus-SMO (5 mg mL⁻¹) and *E. coli* JM101/pSPZ10(StyA-StyB) (5 mg mL⁻¹) were compared by measuring the conversion after 20 min (linearity range for conversion vs. time), under the optimized reaction conditions (section 2.4.7), using styrene (50 mM) as substrate. As the coupling efficiency of both SMOs system is not perfect, formation of H₂O₂ during the reaction is expected. Hence, catalase (2 µM) was added. A set of experiments was conducted under air and O₂ at atmospheric pressure (p_{rel} 0 bar) as well as under pressurized O₂ (p_{rel} 1 to 4 bar). A pressurized closed system (Figure 2.7) might increase the concentration of O₂ in the liquid phase and kinetically enhance the O₂ transfer from the gas phase to the liquid phases.

![Image](image.jpg)

**Figure 2.7.** The oxygen chamber used in this study.

**Figure 2.8** shows that the addition of catalase had a significant influence on the rate of the epoxidation. Independently from the composition and pressure of the gas phase as well as the type of SMO construct, reactions in presence of catalase were accelerated. The chimeric Fus-SMO system performed always better than the bi-enzymatic StyA-StyB system in presence of catalase at any composition and pressure of the gas phase. The maximum rate was obtained with Fus-SMO with pure O₂ at atmospheric pressure (25±2% conversion) with a productivity of 37.5 mM_{product} h⁻¹. Under the same conditions the bi-enzymatic StyA-StyB gave a productivity of 25.5 mM_{product} h⁻¹. Supplying O₂ under pressure was in general
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detrimental for the reaction. The influence of pressure in enzyme catalysis is still a not fully understood phenomenon.\textsuperscript{80-83} According to Le Châtelier’s principle and the Eyring equation, an increase of pressure enhances the rate ($k$) of chemical reactions that have a negative activation volume ($\Delta V^e$).\textsuperscript{84} Considering that typical $\Delta V^e$ values for enzymatic reactions are in the range of $\pm 50$ cm$^3$ mol$^{-1}$, the variation of the reaction rate constant ($\Delta k$) would be less than 1% within the pressure range of our study ($p_{\text{abs}}$ from 1 to 5 bar).\textsuperscript{80,85-87} On the other hand, variation of pressure can have a profound effect in the enzyme structure and, therefore, activity. However, various studies evidenced that significant structural changes in enzyme structure occur at very high pressure, typically above 1 kbar.\textsuperscript{80,85,88,89} Hence, both mentioned effects can be neglected in this study as $O_2$ was supplied at low pressure (max $p_{\text{abs}}$ 5 bar). Thus, we can speculate that the increased $O_2$ pressure in the system may result in an increased formation of FAD-OOH, which cannot be entirely utilised for the epoxidation of styrene. Enhanced rate formation of FAD-OOH generates more $H_2O_2$ that is deleterious for enzyme activity. The addition of catalase can only in part counteract this process as shown in Figure 2.8.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.8.png}
\caption{Conversion [%] of 1a (50 mM) by lyophilized whole cells of \textit{E. coli} BL21 DE3 expressing Fus-SMO (5 mg mL$^{-1}$) and \textit{E. coli} JM101 expressing pSPZ10 (StyA-StyB) (5 mg mL$^{-1}$). Reactions were carried out in glass vials introduced in a sealed pressurized chamber (Figure 2.7). The bio-transformations were performed in a biphasic system KPi (pH 8.0, 50 mM)/n-heptane (1:1 v:v, 1 mL total reaction volume) containing NAD$^+$ (1mM), FAD (50 µM), HCOONa (250 mM, 5 eq.) and Cb-FDH (10 µM). Catalase was added in selected experiments (2 µM). The mixtures were incubated at 30 °C, 200 rpm for 20 min. Two independent experiments were carried out, both in duplicate. Error bars represent the standard deviation.}
\end{figure}
Finally, the higher catalytic activity of the *E. coli*/Fus-SMO cells compared to the *E. coli* cells expressing the bi-enzymatic StyA-StyB was further confirmed in a study in which the conversion of styrene was monitored over time (10, 20 and 30 min; section 2.4.8). *E. coli*/Fus-SMO cells showed a ca. 50% increased catalytic activity compared to *E. coli* cells expressing the bi-enzymatic StyA-StyB (Figure 2.9).

\[
\text{initial activity } Fus_{SMO} - \text{initial activity } StyAStyB \times 100 = 46\%
\]

| Entry | Time [min] | *E. coli* BL21 DE3/Fus-SMO | *E. coli* JM101/pSPZ10-StyA-StyB |
|-------|------------|-----------------------------|---------------------------------|
| 1     | 10         | 10.8±1.7                    | 6.8±1.8                         |
| 2     | 20         | 24.5±1.7                    | 17.7±0.8                        |
| 3     | 30         | 30.0±2.5                    | 20.1±2.8                        |

**Figure 2.9.** Time study for the conversion of 1a (50 mM) by *E. coli* BL21 DE3/Fus-SMO (5 mg mL⁻¹) and *E. coli* JM101/pSPZ10-StyA-StyB (5 mg mL⁻¹). Time range was 0, 10, 20 and 30 minutes. Reactions were carried out in 40 mL glass vials (in a sealed chamber flashed with pure molecular oxygen). The biotransformations were performed in a biphasic system KPi (pH 8.0, 50 mM)/n-heptane (1:1 v:v, 1 mL total reaction volume) containing NAD⁺ (1 mM), FAD (50 µM), HCOONa (250 mM, 5 eq.) and Cb-FDH (10 µM). The mixtures were incubated at 30 °C, 200 rpm up to 30 min. Two independent experiments were carried out and both as duplicate; hence, the reported conversions are the average of 4 measurements. Error bars represent the standard deviations.
2.2.4 Self-sufficient whole cells system for the epoxidation of styrene and derivatives

Aiming at enhancing the practical applicability, a whole cell system was created by expressing simultaneously Fus-SMO and Cb-FDH in E. coli BL21 DE3 as host organism. Under the optimized conditions for the co-expression (i.e. 0.1 mM IPTG at 25 °C for 16 h), the expression for both enzymes in soluble form was observed (Figure 2.10).

![Figure 2.10. SDS-PAGE for the co-expression of Fus-SMO and FDH. Marker: PageRuler™ Unstained Protein Ladder (ThermoFisher Scientific).](image_url)

Preliminary tests demonstrated that lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH were highly active for the epoxidation of 1a and 1b (Table 2.6). It is known from literature that the typical activity of Cb-FDH for the recycling of NADH at the expense of formate is ca. 180 U µmol_{enzyme}^{-1}. 90 Hence, cofactor regeneration is not the rate limiting step. Moreover, the perfect stereoselectivity of Fus-SMO was retained (S)-2a ee >99%; (1S,2S)-2b ee >99% and de >98%.

Table 2.6. Results for the biotransformation of 1a (20 mM) and 1b using lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH.

| Entry | Product         | Conversion [%] | ee [%] | de [%] |
|-------|-----------------|----------------|--------|--------|
| 1     | (S)-2a          | >99            | >99    | n.a.   |
| 2     | (1S,2S)-2b      | 75             | >99    | >98    |

n.a. = not applicable

Then, we monitored the progress of the conversion of 1a and 1b (50 mM) over time under the optimized reaction conditions (Figure 2.11 and section 2.4.9).
Lyophilized whole cells containing Fus-SMO and Cb-FDH (5 mg mL\(^{-1}\)) converted 27.1±1.2% of 1a at 50 mM scale within the first 30 min under air. The epoxidation ran smoothly reaching 93.8±1.3% conversion after 6 h. By prolonging the reaction time, the bio-catalytic system achieved 99% conversion. The epoxidation of 1b showed a similar trend, albeit it proceeded at a lower rate. The reason must be attributed to the lower intrinsic reactivity of SMO towards 1b, as previously shown in Table 2.6. However, a maximum of 90.4±1.4% conversion into (1S,2S)-2b was obtained when starting with substrate 1b (Figure 2.11).

Figure 2.11. Progress of the conversion over the time for the epoxidation of 1a and 1b (50 mM) using lyophilized whole cells wherein Fus-SMO and Cb-FDH where co-expressed in E. coli BL21 DE3 (5 mg mL\(^{-1}\)). Reactions were carried out in sealed 20 mL glass vials under air at atmospheric pressure. The bio-transformations were performed in a biphasic system KPi (pH 8.0, 50 mM)/n-heptane (1:1 v v\(^{-1}\), 1 mL total reaction volume) containing NAD\(^+\) (1 mM), FAD (50 \(\mu\)M), HCOONa (250 mM, 5 eq.) and catalase (2 \(\mu\)M). Two independent experiments were carried out, both in duplicates. Error bars represent the standard deviation (section 2.4.9).

We also investigated the possibility to increase the substrate concentration from 50 mM up to 1 M for the epoxidation of 1a and 1b in analytical scale (total volume 1 mL) using the E. coli BL21 DE3/Fus-SMO/Cb-FDH (5 mg mL\(^{-1}\)) co-expressed system in 20 mL reaction vessels. The results from this study confirmed that nearly quantitative conversion can be achieved at 50 mM substrate concentration applying the current reaction conditions and technical set-up. In fact, the repetition of the epoxidation of 1a and 1b (50 mM) afforded the related products with 98±1 and 96±3 conversions, respectively. Increasing the substrate concentration resulted in a progressive decrease of the conversion (Table 2.7).
Table 2.7. Conversion [%] of 1a (50 mM up to 1M) and 1b (50 mM up to 1 M) to enantiopure 2a and 2b by lyophilized cells of co-expressed Fus-SMO and Cb-FDH in 20 mL glass vials

| Entry | Substrate [mM] | 2a [%] | 2a [mM] | 2b [%] | 2b [mM] |
|-------|---------------|--------|---------|--------|---------|
| 1     | 50            | 98±1   | 49±1    | 96±3   | 48±1    |
| 2     | 75            | 74±2   | 56±2    | 75±1   | 56±1    |
| 3     | 100           | 59±1   | 59±1    | 64±<1  | 64±<1   |
| 4     | 150           | 43±1   | 65±2    | 52±2   | 79±2    |
| 5     | 170           | 34±2   | 58±3    | 51±<1  | 87±1    |
| 6     | 200           | 29<1   | 58±1    | 47±2   | 93±3    |
| 7     | 250           | 24<1   | 59<1    | 39<1   | 98±1    |
| 8     | 500           | 11<1   | 54±2    | 13±1   | 65±3    |
| 9     | 1000          | 5<1    | 48±1    | 5<1    | 50±3    |

Reactions were performed as duplicates; hence, the reported conversion is the average of 2 values. Error is expressed as the absolute difference between the two measurements.

The productivity of the system (i.e. mmol of epoxide product obtained) was partially influenced by the substrate concentration and it depended on the substrate tested. The highest productivity for the epoxidation of 1a was observed at 150 mM substrate concentration, leading to the formation of 65±2 mM of (S)-2a (Table 2.7, entry 4). Instead, the highest productivity for the epoxidation of 1b was observed at 250 mM substrate, leading to the formation of 98±1 mM of (1S,2S)-2b (Table 2.7, entry 7). In addition, we demonstrated that the productivity of the system—applying the current reaction set-up—was not limited by the volume of the gas headspace. In fact, increasing the volume of the reaction vessels up to 100 mL did not improve conversions and productivities (Table 2.8).

Table 2.8. Conversion [%] of 1a (50 mM up to 1M) and 1b (50 mM up to 1M) to enantiopure 2a and 2b by lyophilized cells of co-expressed Fus-SMO and Cb-FDH in 100 mL Erlenmeyer flask

| Entry | Substrate [mM] | 2a [%] | 2a [mM] | 2b [%] | 2b [mM] |
|-------|---------------|--------|---------|--------|---------|
| 1     | 50            | 96±3   | 48±1    | 68±4   | 34±2    |
| 2     | 75            | 52<1   | 39<1    | 54±8   | 40±6    |
| 3     | 100           | 41<1   | 41<1    | 54±7   | 54±7    |
| 4     | 150           | 31<1   | 47<1    | 40<1   | 61±1    |
| 5     | 170           | 29<1   | 50<1    | 39<1   | 66<1    |
| 6     | 200           | 24<1   | 49<1    | 32<2   | 65±3    |
| 7     | 250           | 20<1   | 50±3    | 21<1   | 53<1    |
| 8     | 500           | 12<1   | 58±5    | 13<2   | 67±11   |
| 9     | 1000          | 5<1    | 47±2    | 7<1    | 66±2    |

Reactions were performed as duplicates; hence, the reported conversion is the average of 2 values. Error is expressed as the absolute difference between the two measurements.
Finally, we investigated the influence of different modes of dioxygen supply on the rate of the biocatalytic epoxidation. Hence, experiments were conducted applying either a sealed system with a large volume of air in the headspace or a system with continuous flow of pure dioxygen (bubbling at ca. 1 mL min\(^{-1}\)). In both cases, the other reaction parameters were the same: *E. coli* cells co-expressing Fus-SMO and Cb-FDH (5 mg mL\(^{-1}\)), styrene (50 mM), catalase (2 µM), NAD\(^+\) (1 mM), FAD (50 µM) and HCOONa (5 eq.) in a stirred mixture of aqueous buffer (25 mL, KPi, pH 8.0) and *n*-heptane (25 mL) in round-bottom flasks (section 2.4.9). The difference in the rate of formation of styrene oxide for the two systems was indeed minimal (ca. 10%), with the system consisting of air in the headspace performing better (Figure 2.12). In general, we conclude that sufficient dioxygen supply (independently from the mode) as well as efficient mixing of the biphasic reaction mixture are crucial parameters for sustaining elevated epoxidation rate for longer times.

![Graph comparing sealed and bubbling systems](image)

Relative difference in styrene oxide productivity = \( \frac{0.4552 - 0.4067}{0.4552} \times 100 = 10\% \)

Figure 2.12. Comparing different modes of dioxygen supply: seal system with air headspace (black line) and continuous flow (grey line)

2.2.5 Epoxidation on preparative scale

The bio-catalytic epoxidation using lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH was performed on preparative scale for both substrates 1a
(50 mM, 521 mg) and 1b (50 mM, 591 mg). The reactions were run in a biphasic system KPi (pH 8.0, 50 mM)/n-heptane (1:1 v v⁻¹, 200 mL total reaction volume), under the optimized reaction conditions (section 2.4.10). A large reaction vessel was used in order to assure a sufficient supply of molecular oxygen for the reaction as well as an efficient mixing of the biphasic reaction mixture.

The epoxidation of 1a afforded quantitative conversion (Table 2.9, entry 1). The organic phase was separated from the aqueous buffer and the n-heptane was evaporated, affording 509 mg of (S)-2a (equal to 85% isolated yield) in very high chemical purity (99% measured by GC-FID) as well as optical purity (ee >99 % S). Hence, further purification of (S)-2a obtained from the evaporation of the organic phase was not required. The remaining aliquot of product (S)-2a (ca. 12%) was recovered upon extraction from the aqueous reaction phase. In this case, the purity was determined to be 94%. The by-product was 2-phenyl-ethanol as reported in literature for the natural StyA-StyB enzymatic system. 53, 55, 57, 58, 63 The epoxidation of 1b afforded the product (1S,2S)-2b (Table 2.9, entry 2) in >99% conversion. In this case, the purities of the isolated product from the n-heptane reaction phase and from the extraction of the aqueous phase were similar (ca. 95%). 603 mg of (1S,2S)-2b (90% isolated yield) were obtained with elevated diastereomeric (de >98%) and enantiomeric excess (ee >99%).

Table 2.9 Upscaling for the bio-catalytic synthesis of (S)-2a and (1S,2S)-2b using lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (5 mg mL⁻¹); 200 mL total reaction volume (n-heptane/KPi buffer, 1:1 v v⁻¹).

| Entry | Substrate [mM] | Conversion [%] | Yield [%] | ee [%] | de [%] |
|-------|----------------|----------------|-----------|--------|--------|
| 1     | 1a (50)        | >99            | 85[α]+12  | >99    | -      |
| 2     | 1b (50)        | >99            | 90[β]    | >99    | >98    |

[α] This isolated yield represents the amount of pure product that was isolated from the simple evaporation of the n-heptane as reaction phase. No further work-up was required. The remaining amount of product (ca. 12%) was recovered after extraction from the aqueous reaction phase. However, purity was lower (ca. 94%) due to the generation of 2-phenyl-ethanol as the by-product. [β] In this case, the purities of the isolated product obtained from the n-heptane reaction phase and from the extraction of the aqueous phase were similar. Thus, the aliquots were combined and the total yield was reported.
2.2.6 Determination of the activity of purified Fus-SMO and comparison with literature data of bi-enzymatic StyA-StyB

As chimeric Fus-SMO was created with an N-terminal His₆-tag, the purification was easily performed by Ni²⁺ affinity chromatography (section 2.4.11). We carried out initial determinations of the enzymatic activity of Fus-SMO for the epoxidation of 1a at different pH values. Data of activity showed negligible differences in the range between 6.5 and 9 (data not shown). Therefore, we selected Tris-HCl buffer (50 mM, pH 8.5) for further determination. The determination of the epoxidation activity of Fus-SMO was performed according to the general procedure reported by Otto et al.⁶⁴ and Tischler et al.⁶⁸ for the same experiment with bi-enzymatic StyA-StyB. In this way, the new data of epoxidation activity of Fus-SMO can be compared with the data reported in literature for the two-component StyA-StyB system. Figure 2.13 shows that the specific activities of Fus-SMO and StyA-StyB system are essentially identical for the epoxidation of styrene (1a). However, Fus-SMO showed more than 3-fold increased activity compared to the StyA-StyB system for the epoxidation of para-methylstyrene (1c). We chose substrates 1a and 1c because data of epoxidation activity for two-component StyA-StyB were available in literature. Besides the retained or improved epoxidation activity of Fus-SMO, we point out another important aspect: the conditions for the maximum epoxidation activity of the two-component StyA-StyB is very complicated to reproduce in vitro and nearly impossible in a cell as a 1:1 mixture of StyA and StyB is required (also considering the insolubility of StyB). In contrast, Fus-SMO permits to set effortlessly the highest rate both in vitro as well as in a cell. Finally, comparing the epoxidation activity of our artificial Fus-SMO to natural occurring fused SMOs (StyA2B),⁶⁸ the activity of Fus-SMO was ca. 70-fold and 110-fold higher for 1a and 1c, respectively.
A Chimeric Styrene Monooxygenase with Increased Efficiency in Asymmetric Biocatalytic Epoxidation

| Entry | Substrate | Two-component StyA-StyB [min⁻¹] | Natural fused StyA2B [min⁻¹] | Fus-SMO (this study) [min⁻¹][c] |
|-------|-----------|---------------------------------|-----------------------------|--------------------------------|
| 1     | 1a        | 97ᵃ                              | 1.3ᵇ                         | 95±5                           |
| 2     | 1c        | 14ᵇ                              | 0.4ᵇ                         | 44±4                           |

ᵃRecalculated based on the best activity data (StyA:StyB, 1:1 mol⁻¹) by Otto et al.,ᵇ Recalculated based on best activity data (StyA:StyB, 1:1 mol⁻¹) by Tischler et al.,ᶜ Error is expressed as the standard deviation.

Calculation of the activity of Fus-SMO:

\[
\text{Activity FusSMO} = \left[ \frac{U}{\mu\text{mol FusSMO}} \right] = \frac{\text{Slope}}{100} \times 2 \left[ \frac{\text{mmol product formed}}{\text{min} \times l} \right] \times 10^3 \left[ \frac{\mu\text{mol}}{\mu\text{mol}} \right] \times 3 \left[ \frac{\mu\text{mol FusSMO}}{l} \right]
\]

- Activity of Fus-SMO for epoxidation of 1a

\[
\text{Activity} = \left[ \frac{U}{\mu\text{mol FusSMO}} \right] = \frac{14.2}{100} \times 2 \times 10^3 \times \frac{3}{3} = 95 \text{ (std \ ± 5)}
\]

- Activity of Fus-SMO for epoxidation of 1c

\[
\text{Activity} = \left[ \frac{U}{\mu\text{mol FusSMO}} \right] = \frac{6.6}{100} \times 2 \times 10^3 \times \frac{3}{3} = 44 \text{ (std \ ± 2)}
\]

Figure 2.13. Initial activity purified Fus-SMO for the quantitative formation of enantiopure 2a and 2c. Slope conversion 2a: 14.2±0.8; Slope conversion 2c: 6.6±0.2 Specific activity for the epoxidation of 1a and 1c with Fus-SMO (this study) in Tris-HCl buffer (pH 8.5, 50 mM), two-component StyA-StyB (literature data) and natural fused StyA2B (literature data). For experimental details, see section 2.4.12

Influence of FAD loading and NADH recycling system on the initial activity of purified Fus-SMO

Based on the amount of FAD that is naturally bound to the purified Fus-SMO (ca. 25%), further tests with the purified Fus-SMO system were performed in order to
investigate the influence of FAD loading as well as the possibility to replace stoichiometric amount of NADH with catalytic NAD$^+$ in combination with the FDH recycling system. Again, the initial activity of the catalytic system was examined and styrene 1a was used as model substrate. Four different conditions were tested: in condition A stoichiometric amount of NAD$^+$ and FDH recycling system were used; condition B, same as condition A but without addition of external FAD; condition C, same as condition A but with addition of 0.74 eq. of external FAD; condition D, same as condition A, but with addition of 1.74 eq. of external FAD (for experimental details see section 2.4.12). As shown in Table 2.10, the addition of FAD is of fundamental importance for a better performance of the catalytic system and it is not necessary to be in excess (though it was not detrimental, condition A) in relation to the amount of enzyme, but it is important to be at least in equimolar ratio with the enzyme (1:1 FAD:enzyme) as shown in the results with conditions C. Furthermore, it is possible to avoid using stoichiometric amount of NADH and use instead NAD$^+$ (in this case in stoichiometric amount compared to the substrate, however, it may be possible to go lower) in combination with the NAD$^+$ recycling system (Cb-FDH and HCOONa). Indeed, under these conditions (both A and C) the system performed even better than the standard assay reported above in Figure 2.13.

**Table 2.10. Initial activity of purified Fus-SMO under various conditions**

| Time [min] | Conditions A (NAD$^+$ recycling system and 5 eq. FAD) | Conditions B (NAD$^+$ recycling system and no external FAD) | Conditions C (NAD$^+$ recycling system and 0.74 eq. FAD) | Conditions D (NAD$^+$ recycling system and 1.74 eq. FAD) |
|------------|------------------------------------------------------|-----------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------|
| 0          | 0                                                    | 0                                                         | 0                                                        | 0                                                        |
| 1          | 17                                                   | 10                                                       | 16                                                      | 16                                                      |
| 2          | 29                                                   | 18                                                       | 34                                                      | 25                                                      |
| 3          | 50                                                   | 32                                                       | 46                                                      | 48                                                      |
| Slope      | 16.152                                               | 10.149                                                   | 15.926                                                  | 15.005                                                  |
| R$^2$      | 0.9876                                               | 0.9803                                                   | 0.9948                                                  | 0.9667                                                  |
| Activity   | 108                                                  | 68                                                       | 106                                                     | 100                                                     |

Equivalents NAD$^+$ based on substrate; equivalents FAD based on enzyme
2.2.7 Thermostability of Fus-SMO

The melting temperature of the Fus-SMO was measured in two buffers (Tris-HCl and KPi) at different pH values. Moreover, various additives, such as glycerol, NaCl and FAD were included in the assay in selected experiments. Consistent results and spectra were observed in Tris-HCl buffer from pH 7.0 up to 8.0, while at pH 9.0 two or more peaks were observed; this may indicate protein unfolding. On the other hand, no reliable results were obtained in KPi buffer. The melting temperature in Tris-HCl buffer was 35 °C at pH 7.0 and (39 °C at basic pH (from 7.5 to 8.5). Regarding the influence of the additives in selected experimental conditions, it is not very clear what the real trend is. Apparently, it seems that the pH is of critical importance depending on the additives present. In general, at pH 7 FAD addition stabilizes the enzyme (i.e., melting temperature shifts from 35 °C without any additives to 39 °C), while the sole addition of NaCl as stabilizer has a lower effect than FAD (i.e., shift from 35 °C without additives to 37 °C). On the other hand, at pH 7.5 the additives have no effect on stabilization of the enzyme (i.e., lower Tm observed in all cases) and the highest Tm is detected in absence of additives (39 °C). At pH 8.0, the addition of NaCl increase further the Tm from 39 °C to 41 °C, but FAD does not have any effect and a similar trend is observed at pH 8.5. The best conditions so far turned out to be at pH 8.5 with the addition of NaCl (41 °C). Further experiments disclosed that both glycerol and FAD are beneficial at pH 7.0, while at higher pH values the main influence is given by glycerol albeit the addition of NaCl is also beneficial at higher pH. In summary, the highest Tm was observed in 50 mM Tris-HCl buffer at pH 8.5 when 100 mM NaCl was added (2 °C higher compared with no addition of additives) without the need for other additives.

2.3 Conclusions

In this work, we have created a chimeric styrene monooxygenase (SMO) in which the two enzymatic units (StyA and StyB) are fused via a flexible linker. The fused SMO allowed for solving a few long-standing problems related to its application in chemical synthesis. First, the Fus-SMO was expressed mainly in soluble form whereas recombinant, singular StyB is almost completely insoluble. Second, the activity of StyA and StyB is now properly balanced as they are produced at an exact ratio of 1:1. Third, the flexible linker forces StyB and StyA to be close each other in solution, hence facilitating their contact. Therefore, the channeling transfer of FAD from StyB to StyA might be favored over the diffusive transfer. These properties permit to minimize the waste of reducing equivalents during the overall process.
Moreover, the rates of the biocatalytic epoxidation catalyzed by Fus-SMO was comparable or superior to the value reported in literature for the two-component StyA-StyB. Further comparison with naturally occurring fused SMO systems revealed that our artificial Fus-SMO is about two orders of magnitude more active. Finally, a recent study showed that another artificially fused SMO had more than 5-fold lower epoxidation rate compared to our Fus-SMO. The different behavior must be attributed to the different type of linkers used for the fusion. In our work we have used a longer (i.e. 30 amino acids) and flexible (i.e. containing 70% of glycine) linker, whereas in ref. 72 the linkers were shorter (i.e. from 3 to 6 amino acids) and more rigid (i.e. no glycine residues).

When considering the application of the whole cell system, we can conclude that E. coli cells expressing Fus-SMO possess higher epoxidation activity than E. coli cells expressing separated StyA and StyB as a combination of: i) balanced and improved expression levels of reductase and epoxidase units; ii) intrinsic higher specific epoxidation activity of Fus-SMO.

Another important aspect for future applications in chemical synthesis and biotechnology is that the His6-tagged Fus-SMO can be now easily purified. Single expression of StyA and StyB and, moreover, tedious and low-yielding refolding of StyB are no longer required. Hence, Fus-SMO can now also be applied as isolated enzyme in solution.

Finally, the genes of the Fus-SMO and the formate dehydrogenase (Cb-FDH) were co-expressed in E. coli and applied as a self-sufficient system for the epoxidation in more than 500 milligrams scale of two model substrates: styrene and β-methyl styrene. The epoxide products were isolated in elevated yields and in perfect diasteromerically and enantiomerically pure form. Hence, Fus-SMO retained the exquisite stereoselectivity of the parent bi-enzymatic system.

In summary, this work will open new opportunities in organic synthesis for the exploitation of the asymmetric biocatalytic epoxidation of styrene derivatives. Furthermore, the same concept might be extended to other multi-enzymatic flavin-dependent systems in order to extend the substrate scope of the reaction beyond styrene derivatives.
2.4 Experimental section

**General information.** Styrene (1a, 99.5%), (S)-stylene oxide ((S)-2a, >98%), racemic styrene oxide (rac-2a) and 4-methylstyrene (1c, >99%) were purchased from Fluka Chemicals. trans-β-Methyl styrene (1b, >97) was purchased from TCI Chemicals. (15,25)-1-phenylpropylene oxide (15,25-2b) and (1R,2R)-1-phenylpropylene oxide (1R,2R-2b, 97% purity 99% ee) were purchased from Sigma Aldrich. All chemicals and solvents were used without further purification. Nicotinamide cofactor (NAD+) was purchased from Melford Biolaboratories (Chelsworth, Ipswich, UK). Flavin adenine dinucleotide (FAD) was purchased from TCI Chemicals. Catalase from bovine liver was purchased by Sigma-Aldrich (lyophilized powder, >10000 U mg-1 of protein). Catalase was added to the reactions from a stock solution. The concentration of the catalase in the reaction mixture (ca. 2 µM) was calculated considering the MW of the monomer (60 kDa) since each monomer contains a catalytic iron site. Cb-FDH was expressed and purified as described previously.91

**2.4.1 Expression of E. coli JM101 and E. coli Arctic express cells carrying pSPZ10-StyASTyB**

**JM101:** 800 mL of LB medium supplemented with kanamycin (50 µg mL⁻¹) were inoculated with 15 mL of an overnight culture and grown at 37 °C until an OD₆₀₀ of 0.6-0.8 was reached. Enzyme expression was induced with DCPK (0.05% v v⁻¹), cells were grown for 16 h at 25 °C and then harvested by centrifugation.

**Arctic express DE3:** 800 mL of LB medium supplemented with kanamycin (50 µg mL⁻¹) and gentamycin (20 µg mL⁻¹), were inoculated with 15 mL of an overnight culture and grown at 37 °C until an OD₆₀₀ of 0.6-0.8 was reached. Expression of the proteins was induced by the addition of DCPK (0.05% v v⁻¹). Cells were grown for 16 h at 15 °C and then harvested by centrifugation. Results are summarized in Figure 2.6.

**2.4.2 Activity test: comparison between E. coli Arctic Express/pSPZ10-StyASTyB and E. coli JM101/pSPZ10-StyASTyB**

**Reaction conditions:** Fresh, frozen and lyophilized E. coli cells of JM101 and Arctic Express DE3 containing expressed StyA and StyB (25 °C, 16 h) were subjected to activity tests for the conversion of 1a (10 mM) to 2a as described below:

(a) **JM101**

(1) 3.2 g of wet fresh cells obtained from the expression trials (directly used for bio-catalytic reaction after harvesting of the E. coli culture) were resuspended in 18 mL of KPi buffer (50 mM, pH 7.0, final concentration of wet fresh cells ca. 180 mg mL⁻¹). An aliquot of 0.5 mL was used for each bio-catalytic reaction. (2) Frozen cell pellets were defrosted and the wet cells were resuspended in KPi buffer to obtain a final cell concentration of ca. 130 mg mL⁻¹ (50 mM, pH 7.0). An aliquot of 0.5 mL was used for each biocatalytic reaction (3) Lyophilized
cells (10 mg) were rehydrated in KPi buffer (0.5 mL, 50 mM, pH 7.0) and used for performing the activity check.

(b) Arctic Express DE3
(1) Fresh cells obtained from the expression test (2.3 g) were resuspended in KPi buffer (50 mM, pH 7; 18 mL, final concentration of wet fresh cells ca. 180 mg mL⁻¹). An aliquot of 0.5 mL was used for each test. (2) Frozen cells (2.24 g) were resuspended in KPi buffer (50 mM, pH 7; 18 mL, final concentration of wet cells ca. 130 mL⁻¹). An aliquot of 0.5 mL was used for each bio-catalytic reaction. (3) Lyophilized cells (10 mg) were rehydrated in 0.5 mL KPi buffer (50 mM, pH 7.0) and used for performing the activity check.

Bio-catalytic reactions were performed in a biphasic system (1:1, v/v buffer/decane) in 4 mL glass vials (final reaction volume 1 mL) in the presence of NAD⁺ (1 mM, 0.1 eq.), HCOONa (100 mM, 10 eq.) and Cb-FDH (10 µM) for recycling of the NAD cofactor. The concentrations of coenzyme, co-substrate and recycling enzyme are calculated on the volume of the aqueous phase. The reactions were initiated by the addition of 1a (10 mM, calculated on the volume of the organic phase) and incubated at 30 °C and 170 rpm on an orbital shaker for 16 h. The organic phase reaction mixture (decane) was separated from the aqueous phase. The aqueous phase was further extracted with EtOAc (2x 500 µL). The combined organic phases (decane + EtOAc) were dried with MgSO₄ and the conversions were measured by GC-FID. The results are reported in Table 2.5.

2.4.3 Expression and activity test for the chimeric fused SMO (Fus-SMO) in E. coli BL21 DE3
First expression trial of Fus-SMO
The DNA sequence encoding for styA and styB (GenBank: AF031161.1) was optimized for the expression in E. coli and subcloned in pET28b between Ndel and Xhol to obtain the His₆-StyA-linker-StyB (MW = 69 kDa) construct (flexible linker AA sequence: ASGGGGSGGGGSGGGGSGGGGSGAS).73

When transforming BL21 DE3 cells with the plasmid, colonies of various colors (pink/white/blue colors) grew and four of them were picked and subjected to expression and solubility trials (Figure 2.3). For each colony, 400 mL of LB medium were supplemented with Kanamycin (50 µg/mL) and inoculated with 8 mL of an overnight culture. The cells were grown at 37 °C until the cell density reached a value between 0.6 and 0.8 and split into 100 mL aliquots. To each aliquot IPTG was added (1 mM final concentration) and protein expression was carried out at 37 °C for 3h, 30 °C for 5 h or 25 °C overnight (o.n.). The cells were harvested by centrifugation, washed with KPi buffer (50 mM, pH 8.0) and lyophilized.
Preliminary tests with Fus-SMO as lyophilized whole cells for the conversion of 1a to 2a
Lyophilized cells (20 mg) obtained from the expression test were rehydrated in KPi buffer (50 mM, pH 7.5, 1 mL) in 20 mL glass vials. After that the cofactor NAD⁺ (1 mM, 0.05 eq.) was added followed by addition of HCOONa (100 mM, 5 eq.) and purified Cb-FDH (10 µM). Furthermore, FAD (50 µM) was added to selected experiments. The concentrations of coenzymes, cosubstrate and recycling enzyme are calculated in relation to the volume of the aqueous phase. Decane (1 mL) was used as biphasic solvent and the biotransformations were initiated by the addition of 1a (20 mM, calculated on the volume of the organic phase). The mixtures were shaken at 30 °C and 180 rpm on an orbital shaker for 17 h. The organic phase (decane) was separated from the aqueous phase. The aqueous phase was further extracted with EtOAc (2x 500 µL). The combined organic phases (decane + EtOAc) were dried with MgSO₄ and the conversions were measured by GC-FID and results are reported in Table 2.1.

Optimization of the expression using various concentrations of IPTG and testing the activity of Fus-SMO in BL21 DE3
800 mL of Luria-Bertani Broth were supplemented with kanamycin (50 µg mL⁻¹) and inoculated with 15 mL of an overnight culture of E. coli/pET28b-Fus-SMO (colony 1). The main culture was grown at 37 °C until the cell density reached an OD₆₀₀ of 0.6-0.8. Then, the expression of the protein was induced by the addition of IPTG (0.1, 0.5 and 1 mM, respectively) and the cells grown for additional 16 h at 25 °C and harvested by centrifugation (Figure 2.4).
Activity tests were first performed with the soluble fraction. The activities from all the expression conditions were assayed in order to understand the best condition for expression. Furthermore, the influence of different reaction vessels on the outcome of the biocatalytic reaction was also investigated.

Reaction conditions for the conversion of 1a to 2a by the soluble part of expressed Fus-SMO:
450-500 mg of cells (wet weight) obtained from the expression trials were resuspended in KPi buffer (pH 7.5, 50 mM, 3 mL) and lysed by sonication (5 sec. on, 10 sec. off, 5 min on time, 45 % amplitude). The cell debris was removed by centrifugation and only the supernatant was used for further activity tests as described below. Results are reported in Table 2.3.

Note: the concentrations of coenzymes, cosubstrate and recycling enzyme are always calculated on the volume of the aqueous phase, whereas the concentration of the substrate is referred to the organic phase.
Reactions performed in Eppendorf tubes (2 mL): To a 350 μL aliquot of the soluble fraction, NAD⁺ (1 mM, 0.1 eq.), HCOONa (100 mM, 10 eq.), Cb-FDH (10 μM) and FAD (30 μM) were added (0.5 mL final volume of aqueous phase). Decane (0.5 mL) was used as biphasic solvent and the biotransformation was initiated by the addition of 1a (10 mM). The reaction was shaken at 30 °C and 180 rpm on an orbital shaker for 20 h. The organic phase from the reaction mixture (decane) was separated from the aqueous phase. The aqueous phase was further extracted with EtOAc (2x 250 μL). The combined organic phases (decane + EtOAc) were dried with MgSO₄ and the conversions were measured by GC-FID.

Reactions performed in 4 mL glass vials: To a 350 μL aliquot of the soluble fraction, NAD⁺ (1 mM, 0.1 eq.), HCOONa (100 mM, 10 eq.), Cb-FDH (10 μM) and FAD (30 μM) were added (0.5 mL final volume of aqueous phase). Decane (0.5 mL) was used as biphasic solvent and the biotransformation was initiated by the addition of 1a (10 mM). The mixture was shaken at 30 °C and 180 rpm on an orbital shaker for 20 h. The organic phase from the reaction mixture (decane) was separated from the aqueous phase. The aqueous phase was further extracted with EtOAc (2x 250 μL). The combined organic phases (decane + EtOAc) were dried with MgSO₄ and the conversions were measured by GC-FID.

Reactions performed in 20 mL glass vials: To a 700 μL aliquot of the soluble fraction, NAD⁺ (1 mM, 0.1 eq.), HCOONa (100 mM, 10 eq.), Cb-FDH (10 μM) and FAD (30 μM) were added (1 mL final volume of aqueous phase). Decane (1 mL) was used as biphasic solvent and the biotransformation was initiated by the addition of 1a (10 mM). The mixture was shaken at 30 °C and 180 rpm on an orbital shaker for 20 h. The organic phase from the reaction mixture (decane) was separated from the aqueous phase. The aqueous phase was further extracted with EtOAc (2x 500 μL). The combined organic phases (decane + EtOAc) were dried with MgSO₄ and the conversions were measured by GC-FID.

2.4.4 Reaction conditions for the conversion of 1a to (S)-2a by lyophilized whole cells containing Fus-SMO

The reactions were performed in 4 mL glass vials, as this type of vessel showed highest conversion in the preliminary test (see above). Lyophilized cells obtained from the expression trial using various concentration of IPTG for induction were tested for the activity towards the conversion of 1a. Also, the amount of lyophilized cells that is needed for the conversion of 20 mM substrate was investigated. Results are reported in Table 2.2.

Lyophilized cells (2.5, 5 and 10 mg) were rehydrated in KPi buffer (0.5 mL, 50 mM, pH 7.5) in 4 mL glass vials. After that the cofactor NAD⁺ (1 mM, 0.05 eq.) was added followed by addition of HCOONa (100mM, 5 eq.), Cb-FDH (10 μM) and FAD (50 μM). The concentrations of coenzymes, cosubstrate and recycling enzyme are calculated on the volume of the
aqueous phase. Decane (0.5 mL) was used as biphasic solvent and the biotransformation was initiated by the addition of 1a (20 mM, referred to the organic phase). The mixture was shaken at 30 °C and 180 rpm on an orbital shaker for 17 h. The organic phase from the reaction mixture (decane) was separated from the aqueous phase. The aqueous phase was further extracted with EtOAc (2x 250 μL). The combined organic phases (decane + EtOAc) were dried with MgSO₄ and the conversions were measured by GC-FID.

### 2.4.5 Solvent screening: bioconversion of 1a to (S)-2a by lyophilized cells containing Fus-SMO

For all experiments, cells were used after expression with 0.1 mM IPTG at 25 °C for 16 h.

**Note:** The concentrations of coenzymes, co-substrate and recycling enzyme are always calculated on the volume of the aqueous phase, the concentration of the substrate is referred to the organic phase and results are reported in Table 2.4.

#### 1:1 ratio n-hexane or n-heptane/KPi buffer

Lyophilized whole cells (10 mg) were rehydrated in KPi buffer (0.5 mL, 50 mM, pH 8) in 4 mL glass vials. After that, the cofactor NAD⁺ (1 mM, 0.05 eq.) HCOONa (100 mM, 5 eq.), Cb-FDH (10 μM) and FAD (50 μM) were added. Two organic solvents (n-hexane or n-heptane; 1:1, v/v with the buffer) were tested as biphasic solvents. Finally, the biotransformations were initiated by the addition of 1a (20 mM). The reactions were shaken at 30 °C and 180 rpm on an orbital shaker for 24 h. The organic phase from the reaction mixture was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (2x 250 μL). The combined organic phases were dried with MgSO₄ and the conversions were measured by GC-FID.

#### KPi buffer

Lyophilized whole cells (20 mg) were rehydrated in KPi buffer (1 mL, 50 mM, pH 8) in 4 mL glass vials. After that the cofactor NAD⁺ (1 mM, 0.05 eq.), HCOONa (100 mM, 5 eq.), Cb-FDH (10 μM) and FAD (50 μM) were added. The biotransformations were initiated by the addition of 1a (20 mM). The reactions were shaken at 30 °C and 180 rpm on an orbital shaker for 24 h. The organic compounds were extracted with MTBE (2x 250 μL), the combined organic layers were dried over MgSO₄ and the conversions were measured by GC-FID.

#### 5% styrene or 2-10% decane

Lyophilized whole cells were rehydrated in KPi buffer (50 mM, pH 8.0) in 4 mL glass vials. Then, the cofactor NAD⁺ (0.1 eq), HCOONa (5 eq.), Cb-FDH (10 μM) and FAD (50 μM) were added. In selected experiments, decane was used (2-10% v/v; 1 mL total reaction volume) as second phase or styrene (5% v/v; 1 mL total reaction volume) as neat substrate. The bio-transformations were initiated by the addition of 1a (10 – 20 μmol) in the case of decane (2 to 10 % v/v) as organic solvent or 1a (435 mM) in the case of styrene used as neat substrate. The reactions were shaken at 30 °C and 180 rpm on
an orbital shaker for 24 h. The organic phase from the reaction mixture was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (2 x 400-500 µL). The combined organic layers were dried over MgSO₄ and the conversions were measured by GC-FID.

2.4.6 Determination of the required reducing equivalents (i.e. equivalents of HCOONa) for the bio-conversion of 1a (20 mM) to (S)-2a using lyophilized whole cells containing Fus-SMO

Lyophilized whole cells of overexpressed Fus-SMO (5 mg, 10 mg mL⁻¹) were rehydrated in KPi buffer (0.5 mL, 50 mM, pH 8) in 4 mL glass vials. NAD⁺ (1 mM, 0.05 eq.), HCOONa (0-100 mM, 0-5 eq.), Cb-FDH (10 µM), FAD (50 µM), n-heptane (0.5 mL) and 1a (20 mM, referred to organic phase) were added and the reactions were incubated at 30 °C and 180 rpm on an orbital shaker for 24 h. The concentrations of coenzymes, cosubstrate and recycling enzyme are calculated on the volume of the aqueous phase. The organic phase from the reaction mixture (n-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (2x 250 µL). The combined organic phases (n-heptane + MTBE) were dried with MgSO₄ and the conversions were measured by GC-FID (Table 2.11).

Table 2.11. Conversions [%] of 1a (20 mM) to (S)-2a by Fus-SMO (10 mg mL⁻¹) and various concentrations of hydride donor

| Entry | HCOONa [mM] | eq. HCOONa | (S)-2a [%][a] |
|-------|-------------|------------|---------------|
| 1     | 0           | 0          | 21±8          |
| 2     | 2           | 0.1        | 22±3          |
| 3     | 4           | 0.2        | 19±1          |
| 4     | 8           | 0.4        | 28±1          |
| 5     | 12          | 0.6        | 23±0          |
| 6     | 16          | 0.8        | 34±1          |
| 7     | 20          | 1          | 37±6          |
| 8     | 40          | 2          | 63±1          |
| 9     | 60          | 3          | 93±1          |
| 10    | 80          | 4          | 99±1          |
| 11[b] | 100         | 5          | 99±1          |

[a] Reactions were performed in duplicates and the reported conversion is the average of two measurements. [b] The optical purity of the final product was measured by HPLC on a chiral column for sample entry 11. The enantiomeric excess was > 99% (S).
2.4.7 Influence of dioxygen in the headspace on the performance of Fus-SMO (expressed in E. coli BL21 DE3) and natural StyA-StyB (expressed in E. coli JM101, plasmid pSPZ10)

Effect of oxygen pressure on the bio-catalytic conversion of 1a (50 mM) in a pressurizable chamber

Lyophilized E. coli cells containing Fus-SMO or natural StyA-StyB (pSPZ10 plasmid) (2.5 mg; 5 mg mL⁻¹) were rehydrated in KPi buffer (pH 8, 50 mM, 0.5 mL) in 20 mL glass vials. The buffer already contained the required amount of NAD⁺ (1 mM, 0.02 eq.), HCOONa (250 mM, 5 eq.), FAD (50 µM) and Cb-FDH (10 µM). In certain experiments catalase (2 µM) was also added. The biotransformations were started by addition of n-heptane (0.5 mL) as the second solvent phase followed by the addition of 1a (50 mM; calculated on the volume of the organic phase). The mixtures were shaken in a closed chamber (Figure 2.7) on an orbital shaker (200 rpm) at 30 °C for 20 minutes. The reactions were quenched by freezing in liquid nitrogen. The organic phase from the reaction mixture (n-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (500 µL). The combined organic phases (n-heptane + MTBE) were dried with MgSO₄ and the conversions were measured by GC-FID (Table 2.12 and 2.13).

**Table 2.12.** Conversion [%] of 1a (50 mM) to (S)-2a using lyophilized cells containing Fus-SMO (5 mg mL⁻¹) in a closed chamber. Reaction time: 20 minutes.

| Entry | O₂ pressure [bar][a] | E. coli BL21 DE3/Fus-SMO 2a [%][c] | E. coli BL21 DE3/Fus-SMO/catalase[b] 2a [%][c] |
|-------|----------------------|------------------------------------|-----------------------------------------------|
| 1     | Atmospheric pressure | 16.3±0.6                           | 21.2±2.9                                      |
| 2     | Saturation with pure O₂ | 10.0±1.0                           | 24.5±1.7                                      |
| 3     | 1                    | 10.8±1.0                           | 20.4±2.2                                      |
| 4     | 2                    | 9.1±2.9                            | 18.6±1.1                                      |
| 5     | 3                    | 10.0±1.0                           | 17.1±1.4                                      |
| 6     | 4                    | 7.8±1.8                            | 14.6±2.9                                      |

[a] Experiments were performed in a sealed pressurized chamber; for entry 2 the chamber was saturated with pure O₂ before reactions were started; [b] final catalase concentration 2 µM; [c] Reactions were performed in duplicates and two independent experiments were performed; thus, the reported conversion is then the average of the 4 values. Errors are expressed as standard deviation.
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Table 2.13. Conversion [%] of 1a (50 mM) to (S)-2a using lyophilized cells containing natural StyA and StyB (5 mg mL\(^{-1}\)) in a closed chamber. Reaction time: 20 minutes.

| Entry | O\(_2\) pressure [bar]\(^[a]\) | \(E.\) coli JM101/pSPZ10-\(\text{StyAS}\)\(\text{StyB}\) \(2\)a [%]\(^[c]\) | \(E.\) coli JM101/pSPZ10-\(\text{StyAStyB}\) \(2\)a [%]\(^[c]\) |
|-------|-----------------|-----------------|-----------------|
| 1     | Atmospheric pressure | 11.8±2.3 | 14.0±2.6 |
| 2     | Saturation with pure O\(_2\) | 13.0±0.1 | 17.7±0.8 |
| 3     | 1                | 11.8±3.5 | 12.6±2.0 |
| 4     | 2                | 8.4±2.1 | 9.6±0.4 |
| 5     | 3                | 8.3±0.7 | 9.0±0.7 |
| 6     | 4                | 6.4±1.2 | 6.3±0.6 |

\(^[a]\) Experiments were performed in a sealed pressurized chamber; for entry 2 the chamber was saturated with pure O\(_2\) before reactions were started; \(^[b]\) final catalase concentration 2 µM; \(^[c]\) reactions were performed in duplicates and two independent experiments were performed; thus, the reported conversion is then the average of the 4 errors. Errors are expressed as standard deviation.

2.4.8 Final comparison of the catalytic efficiency between the chimeric Fus-SMO and the natural StyA-StyB system (from plasmid pSPZ10)

Lyophilized \(E.\) coli cells carrying Fus-SMO or natural StyA-StyB (plasmid pSPZ10) (2.5 mg; 5 mg ml\(^{-1}\)) were rehydrated in KPi buffer (pH 8.0, 50 mM, 0.5 mL) in 20 mL glass vials. The buffer already contained the required amount of NAD\(^+\) (1mM, 0.02eq.), HCOONa (250 mM, 5 eq.), FAD (50 µM), Cb-FDH (10 µM) and catalase (2 µM). The biotransformations were started by the addition of \(n\)-heptane (0.5 mL) as the second solvent phase followed by the addition of 1a (50 mM; calculated on the volume of the organic phase). The mixtures were shaken in a closed chamber on an orbital shaker (200 rpm) at 30 °C for 10, 20 and 30 minutes. The reactions were quenched by freezing in liquid nitrogen. The organic phase from the reaction mixture (\(n\)-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (500 µL). The combined organic phases (\(n\)-heptane + MTBE) were dried with MgSO\(_4\) and the conversions were measured by GC-FID.

2.4.9 Co-expression of Fus-SMO and Cb-FDH in \(E.\) coli BL21 DE3

LB medium (800 mL), supplemented with kanamycin (50 µg mL\(^{-1}\)) and ampicillin (100 µg mL\(^{-1}\)), was inoculated with 15 mL of a pre-culture and grown at 37 °C until the cell density reached an OD\(_{600}\) value of 0.6-0.8. The expression was induced by the addition of IPTG (0.1 mM final concentration) and the cells were grown for further 16 h at 25 °C prior to harvesting by centrifugation at 4500 rpm. The whole cells were then washed with KPi buffer (pH 8, 50 mM), lyophilized (ca. 2g dry weight) and stored at -20 °C (Figure 2.10).

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Activity test for the co-expressed cells

Lyophilized whole cells containing Fus-SMO and Cb-FDH (5 mg) were rehydrated in KPi buffer (pH 8, 50 mM, 0.5 mL) in 4 mL glass vials. NAD$^+$ (1 mM, 0.05 eq.), FAD (50 µM), HCOONa (100 mM, 5 eq.) and heptane (0.5 mL) were added. The biotransformations were started by the addition of substrate (1a or 1b, 20 mM) and shaken at 30 °C, 180 rpm on an orbital shaker for 24 h. The concentrations of coenzymes, co-substrate and recycling enzyme are always calculated on the volume of the aqueous phase, the concentration of the substrate is referred to the organic phase. The organic phase from the reaction mixture (n-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (2 x 250 µL). The combined organic phases (n-heptane + MTBE) were dried with MgSO$_4$ and analyzed by GC-FID and chiral HPLC (Table 2.6).

Time study for the bio-catalytic epoxidation of 1a and 1b (50 mM) to enantiopure (S)-2a and (1S,2S)-2b using lyophilized whole cells of co-expressed Fus-SMO and Cb-FDH.

Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (2.5 mg, 5 mg mL$^{-1}$) were rehydrated in KPi buffer (pH 8, 50 mM, 0.5 mL) in a 20 mL glass vial. NAD$^+$ (1 mM), FAD (50 µM), HCOONa (5 eq.), catalase (2 µM), n-heptane (0.5 mL) and the substrates 1a (50 mM) or 1b (50 mM) were added. The concentration of cells, coenzyme and recycling enzyme are calculated on the volume of the aqueous phase, while the concentration of the substrate on the organic phase. The reactions were incubated at 30 °C and 200 rpm on an orbital shaker and monitored over time (0.5, 1, 2, 3, 4, 5, 6 and 24 h). The reactions were quenched by freezing in liquid nitrogen. The organic phase from the reaction mixture (n-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (500 µL). The combined organic phases (n-heptane + MTBE) were dried with MgSO$_4$. The conversions were analyzed by GC-FID (Table 2.14).
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Table 2.14. Time study for the conversion of 1a and 1b (50 mM) by lyophilized cells of co-expressed Fus-SMO and Cb-FDH

| Entry | Time [h] | (S)-2a [%] | (15,25)-2b [%] |
|-------|----------|------------|---------------|
| 1     | 0.5      | 27.1±1.2   | 17.6±0.5      |
| 2     | 1        | 51.4±5.3   | 35.8±1.6      |
| 3     | 2        | 70.9±3.4   | 57.4±0.5      |
| 4     | 3        | 83.4±4.3   | 68.6±1.8      |
| 5     | 4        | 86.6±3.4   | 75.4±3.0      |
| 6     | 5        | 91.8±2.5   | 81.1±2.2      |
| 7     | 6        | 93.8±1.3   | 82.5±1.6      |
| 8     | 24       | 98.8±1.2   | 90.4±1.4      |

Reactions were performed as duplicates in two independent experiments; hence, the reported conversion is the average of 4 values. Error is expressed as the standard deviation.

Experiments with increased substrate concentration for the bio-epoxidation of 1a and 1b (50 mM - 1 M) using lyophilized E. coli cells co-expressing Fus-SMO/Cb-FDH

General procedure. Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (2.5 mg, 5 mg mL⁻¹) were rehydrated in KPi buffer (pH 8, 50 mM, 0.5 mL) in a 20 mL glass vial. NAD⁺ (1 mM), FAD (50 µM), HCOONa (5 eq.), catalase (2 µM), n-heptane (0.5 mL) and the substrates 1a (from 50 to 1 M) or 1b (from 50 mM to 1 M) were added. The concentration of cells, coenzyme and recycling enzyme are calculated on the volume of the aqueous phase, while the concentration of the substrate is calculated on the volume of on the organic phase. The reactions were incubated at 30 °C and 200 rpm on an orbital shaker for 24 h. The organic phase from the reaction mixture (n-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (500 µL). The combined organic phases (n-heptane + MTBE) were dried with MgSO₄. The conversions were analyzed by GC-FID (Table 2.7).

General procedure. Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (5 mg, 5 mg mL⁻¹) were rehydrated in KPi buffer (pH 8, 50 mM, 1 mL) in a 100 mL Erlenmeyer flask. NAD⁺ (1 mM), FAD (50 µM), HCOONa (5 eq.), catalase (2 µM), n-heptane (1 mL) and the substrates 1a (from 50 mM to 1 M) or 1b (50 mM to 1 M) were added. The concentration of cells, coenzyme and recycling enzyme are calculated on the volume of the aqueous phase, while the concentration of the substrate is calculated on the volume of the organic phase. The reactions were incubated at 30 °C and 200 rpm on an orbital shaker for 24 h. The organic phase from the reaction mixture (n-heptane) was separated from the aqueous phase. The aqueous phase was further extracted with MTBE (2 x 500 µL). The combined organic phases (n-heptane + MTBE) were dried with MgSO₄. The conversions were analyzed by GC-FID (Table 2.8).
Study on the influence of the mode of dioxygen transfer in the biphasic biocatalytic epoxidation: sealed system with air headspace versus system with continuous flow (i.e. bubbling) of dioxygen

General procedure for the epoxidation applying a sealed system with air headspace:
Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (125 mg, 5 mg mL⁻¹) were rehydrated in KPi buffer (pH 8, 50 mM, 25 mL) in a 500 mL three necks round-bottom flask. NAD⁺ (1 mM), FAD (50 μM), HCOONa (5 eq.), catalase (2 μM), n-heptane (25 mL) and the substrate 1a (50 mM) were added. The concentration of cells, coenzyme and recycling enzyme are calculated on the volume of the aqueous phase, while the concentration of the substrate is calculated on the volume of the organic phase. A set of reactions was conducted at room temperature under magnetic agitation for 30 min, 45 min, 60 min and 24 h. A vigorous agitation (ca. 700-800 rpm) was set during the reaction in order to obtain an emulsion without a visible separation between the aqueous phase and the organic phase. In this way, we can minimize the mass transfer resistance of styrene from the organic phase to the aqueous phase as well as of styrene oxide in the contrary direction. Thus, any possible kinetic limitation in the mass transfer of dioxygen from the gas phase to the liquid phases can be studied under these conditions.

The rate of the epoxidation reaction expressed as mass of styrene oxide produced per minute is reported in **Figure 2.12** (black line).

General procedure for the epoxidation applying continuous flow of dioxygen (bubbling):
Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (125 mg, 5 mg mL⁻¹) were rehydrated in KPi buffer (pH 8, 50 mM, 25 mL) in a 100 mL three necks round-bottom flask. NAD⁺ (1 mM), FAD (50 μM), HCOONa (5 eq.), catalase (2 μM), n-heptane (25 mL) and the substrate 1a (50 mM) were added. The concentration of cells, coenzyme and recycling enzyme are calculated on the volume of the aqueous phase, while the concentration of the substrate is calculated on the volume of the organic phase. The flow of molecular oxygen was supplied to the reaction via a needle placed through a septum in one neck of the round-bottom flask. That permitted the bubbling of pure dioxygen (ca. 1 mL min⁻¹) directly into the reaction mixture. Another small needle was placed through another septum in the flask in order to prevent any overpressure. A set of reactions was conducted at room temperature under magnetic agitation for 30 min, 45 min, 60 min and 24 h. A vigorous agitation (ca. 700-800 rpm) was set during the reaction in order to obtain an emulsion without a visible separation between the aqueous phase and the organic phase. In this way, we can minimize the mass transfer resistance of styrene from the organic phase to the aqueous phase as well as of styrene oxide in the contrary direction. Thus, any possible kinetic limitation in the mass transfer of dioxygen from the gas phase to the liquid phases can be studied under these conditions.

The rate of the epoxidation reaction expressed as mass of styrene oxide produced per minute is reported in **Figure 2.12** (grey line).
General optimized procedure for the bio-catalytic synthesis of (S)-2a and (1S,2S)-2b by lyophilized whole cells co-expressing Fus-SMO and Cb-FDH (analytical scale). Lyophilized whole cells (5 mg) were rehydrated in KPi buffer (0.5 mL, 50 mM, pH 8) in 4 mL or 20 mL glass vials, containing NAD\(^+\) (1 mM), HCOONa (5 eq.), FAD (50 µM) and catalase (2 µM). n-Heptane (0.5 mL) and the substrate 1a-b (50 mM) were added. The concentration of the substrate is calculated on the volume of the organic phase. The concentrations of NAD\(^+\), HCOONa are calculated on the volume of the aqueous phase. The mixture was incubated at 30 °C and 180 rpm on an orbital shaker. At the end of the reaction, the organic phase was separated from the aqueous phase. The aqueous phase was extracted with MTBE (2 x 250 µL). The combined organic phases were dried with MgSO\(_4\). The conversions were measured by GC-FID, whereas the ee and de were measured by HPLC.

2.4.10 Preparative scale bio-catalytic epoxidation

General optimised procedure for the bio-catalytic synthesis of (S)-2a by whole cells co-expressing Fus-SMO and Cb-FDH (scale up). Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (500 mg, 5 mg mL\(^{-1}\)) were rehydrated in KPi buffer (pH 8, 50 mM, 100 mL) in a 1 L tri-baffled flask. NAD\(^+\) (1 mM), FAD (50 µM), HCOONa (5 eq.) and catalase (2 µM) were added. n-Heptane (100 mL) and the substrate 1a (50 mM, 521 mg, 5.00 mmol) were added. The concentration of cells, NAD\(^+\) and HCOONa are calculated on the volume of the aqueous phase. The concentration of the substrate is calculated on the volume of the organic phase. The reaction was incubated at 30 °C and 200 rpm on an orbital shaker for 16 h. After completion of the reaction confirmed by GC-FID, the n-heptane phase was recovered, dried with MgSO\(_4\) and evaporated under reduced pressure yielding 509 mg (85%) of (S)-2a (99% purity by GC-FID; ee >99% by chiral HPLC. Separately, the aqueous phase was extracted with MTBE (2 x 50 mL); the organic layer was dried with MgSO\(_4\) and the solvent was evaporated under reduced pressure, affording the remaining 12% of product in 94% chemical purity.

General optimized procedure for the bio-catalytic synthesis of (1S,2S)-2b by lyophilized whole cells co-expressing Fus-SMO and Cb-FDH (scale-up). A similar procedure was performed as reported above. Lyophilized whole cells containing co-expressed Fus-SMO and Cb-FDH (500 mg, 5 mg mL\(^{-1}\)) were rehydrated in KPi buffer (pH 8, 50 mM, 100 mL) in a 1 L tri-baffled flask. NAD\(^+\) (1 mM), FAD (50 µM), HCOONa (5 eq.) and catalase (2 µM) were added. n-Heptane (100 mL) and substrate 1b (50 mM, 591 mg, 5.00 mmol) was added. The concentration of cells, NAD\(^+\) and HCOONa are calculated on the volume of the aqueous phase. The concentration of the substrate is calculated on the volume of the organic phase. The reactions were incubated at 30 °C and 200 rpm on an orbital shaker for 16 h. After completion of the reaction confirmed by GC-FID, the n-heptane phase was recovered and evaporated under reduced pressure. The aqueous phase was then extracted with MTBE (2 x 50 mL). In this case, the purity of the product (1S,2S)-2b was similar in the n-heptane phase.
and in the extracted MTBE phase. Thus, the organic phases were combined, dried with MgSO₄ and the solvent was evaporated. The final yield was 603 mg of (1S,2S)-2b (90% isolated yield, 95% purity by GC-FID; ee >99% and de >98% by chiral HPLC).

2.4.11 Purification His₆ Fus-SMO

Expression was conducted as previously described in section 2.4.3 (0.1 mM IPTG, 25 °C overnight).

Wet cells containing Fus-SMO (ca. 12 g) were resuspended in lysis buffer (ca. 65 mL; 50 mM KH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8) and disrupted by sonication (10 min, amplitude 45%, pulse on 10 s, pulse off 10 s). The suspension was centrifuged (18000 rpm, 1 h, 4 °C). The supernatant was filtered through a 0.45 μm filter and loaded onto a Ni²⁺ column (5 mL, GE Healthcare) that was previously conditioned with lysis buffer. The column was washed with washing buffer (50 mM KH₂PO₄, 300 mM NaCl, 25 mM imidazole, pH 8) and the protein eluted with elution buffer (50 mM KH₂PO₄, 300 mM NaCl, 300 mM imidazole, pH 8). Purity was analyzed by SDS-Page and fractions showing >95% purity were combined and dialyzed overnight against potassium phosphate buffer (6 L, pH 8, 20 mM). The enzyme solution was concentrated and the concentration was measured spectrophotometrically by the method of Bradford (Bovine Serum Albumine standard). 128 mg of protein were obtained from ca. 12 g of wet cells, equal to 3.2 L of culture, without further optimization. The purified protein shows a yellow color which corresponds to a natural loading of FAD of ca. 25%. The loading of FAD was analyzed by UV-Vis-spectroscopy at 450 nm by using the characteristic extinction coefficient of free FAD (ε=11300 M⁻¹ cm⁻¹).

2.4.12 Typical assay for the determination of the initial activity of purified His₆ Fus-SMO

The epoxidation activity of Fus-SMO with 1a and 1c was measured according to the procedure reported by Otto et al.⁶⁴ as well as Tischler et al.⁶⁸ The quantification of the product formation vs time was performed by quenching samples at different time points and determining the conversion by GC-FID. A typical sample assay contained (2 mL Eppendorf tube): Fus-SMO (3 μM), FAD (15 μM), HCOONa (150 mM), catalase (650 U), Cb-FDH (20 μM), styrene (1a) or 4-methylstyrene (3a) (2 mM, from a 50 mM stock solution in heptane) in a 1 mL total volume of Tris-HCl buffer (pH 8.5, 50 mM). The mixture was incubated at 37 °C for 1 min and the reaction was started by the addition of NADH (50 mM). The samples were shaken at 37 °C, 1400 rpm for 1, 2, 3, 4 and 5 min, respectively. Reactions were stopped by freezing in liquid nitrogen followed by extraction with MTBE (2 x 500 μL). The organic phase was separated from the aqueous layer, dried over MgSO₄ and injected into GC-FID.
Influence of FAD loading and NADH recycling system on the initial activity of purified Fus-SMO: The same assay as described above was used for these tests with some variations as follow:

**CONDITION A:** Fus-SMO (3 μM), FAD (15 μM, 5 eq. based on Fus-SMO), NAD\(^+\) (2 mM, 1 eq. based on 1a), HCOONa (150 mM), catalase (650 U), Cb-FDH (60 μM), styrene (1a) (2 mM, from a 50 mM stock solution in heptane) in a 1 mL total volume of Tris-HCl buffer (pH 8.5, 50 mM).

**CONDITION B:** Fus-SMO (3 μM), NAD\(^+\) (2 mM, 1 eq. based on 1a), HCOONa (150 mM), catalase (650 U), Cb-FDH (60 μM), styrene (1a) (2 mM, from a 50 mM stock solution in heptane) in a 1 mL total volume of Tris-HCl buffer (pH 8.5, 50 mM).

**CONDITION C:** Fus-SMO (3 μM), FAD (2.22 μM, 0.74 eq. based on Fus-SMO), NAD\(^+\) (2 mM, 1 eq. based on 1a), HCOONa (150 mM), catalase (650 U), Cb-FDH (60 μM), styrene (1a) (2 mM, from a 50 mM stock solution in heptane) in a 1 mL total volume of Tris-HCl buffer (pH 8.5, 50 mM).

**CONDITION D:** Fus-SMO (3 μM), FAD (5.22 μM, 1.74 eq. based on Fus-SMO), NAD\(^+\) (2 mM, 1 eq. based on 1a), HCOONa (150 mM), catalase (650 U), Cb-FDH (60 μM), styrene (1a) (2 mM, from a 50 mM stock solution in heptane) in a 1 mL total volume of Tris-HCl buffer (pH 8.5, 50 mM).

2.4.13 Thermostability measurement

The thermostability (melting temperature \(T_m\), defined as the temperature at which 50% of the enzyme unfolds) of Fus-smo was measured in 50 mM Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5 and 9.0) as well as in 50 mM KPi buffer (pH 6.0, 6.5, 7.0, 7.5 and 8.0) by differential scanning fluorometry using a Biorad-7500 QPCR machine. Fluorescence data were collected as a continuous standard melt curve from 20-90 °C (1% increment, hold 1 min at 20 °C and 1 min at 90 °C), using ROX for reporter and none for quencher. Also, none was selected as passive reference. A standard sample (20 μL total final volume per well) contained the following components: Fus-SMO (1 μg), buffer (50 mM), NaCl (100 mM), Sypro Orange (5 times), Glycerol (5% vol/vol), FAD (10.7 μM) and MilliQ.

**Note:** Buffer, Sypro Orange and Milli-Q were always present in the mixture while all the other components (additives) were varied as shown in Table 2.15.
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2.4.14 Analytical methods

**GC-FID method A: determination of the conversion** Column: Agilent J&W DB1701 (30 m, 250 µm, 0.25 µm). Carrier gas: H₂; Parameter: T injector 250 ºC; constant pressure 14.50 psi; temperature program: 80 ºC, hold 6.5 min; gradient 10 ºC min⁻¹ up to 160 ºC, hold 5 min; gradient 20 ºC min⁻¹ up to 200 ºC, hold 2 min; gradient 20 ºC min⁻¹ up to 280 ºC, hold 1 min

**NP-HPLC method B: determination of the enantiomeric excess (ee) and diastereomeric excess (de)** Column: Daicel Chiralcel OD (0.46 cm x 25 cm); HPLC program: constant oven
temperature 25 °C; eluent composition: isocratic Hexane/Isopropanol 99:1; flow rate: 0.5 mL min⁻¹

Table 2.17. Retention time [min] of substrates and products used in this study

| Compound | Retention time [min] |  | Retention time [min] |
|----------|-----------------------|----------------|-----------------------|
|          | GC-FID                | HPLC          |                       |
| 1a       | 4.9                   | n.a.          |                       |
| (S)-2a   | 10.9                  | 15.9          |                       |
| 1b       | 8.9                   | n.a.          |                       |
| (15S)-2b | 11.9                  | 16.1          |                       |

n.a = not applicable; The absolute configuration of the products (S)-2a and (15S)-2b were identified by comparison with authentically optically active reference compounds.

2.5 References

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