Enhanced Semi-Preparative Biotransformation of Cumene Dioxygenase: From Analytical Scale to Product Isolation

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Supporting Information available online

Dedicated to Prof. Dr. Christian Wandrey on the occasion of his 80th birthday

Scale-up of oxygenase catalyzed reactions is often challenging due to the limited oxygen mass transfer in aqueous solutions. To overcome such limitation, we studied different scale-up conditions using recombinant resting cells of *E. coli* JM109(DE3), harboring the cumene dioxygenase of *Pseudomonas fluorescens* IP01, for the dihydroxylation of naphthalene to (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol. Thereby, vigorous stirring of the biotransformation in a 2 L round bottom flask in combination with an oxygen-enriched headspace exhibited outstanding product formation after 1 h. Furthermore, the enhanced setup was used for the cumene dioxygenase catalyzed biosynthesis of 240 mg of valuable (+)-*trans*-carveol from (*R*)-(+)-limonene, demonstrating the application of our workflow for volatile compounds.

Keywords: Biocatalysis, Cumene dioxygenase, Hydroxylation, Rieske non-heme iron oxygenases, Scale-up

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1 Introduction

In research laboratories, biocatalytic reactions are often operated on analytical scale generating low amounts of product, only detectable by HPLC, GC or indirect assays [1]. Thereby, products are identified by comparison of retention times and fragmentation patterns with commercially available standards, self-synthesized standards or by comparison with databases. To obtain the product of a biotransformation in larger quantity, for example for the structural elucidation of novel compounds, semi-preparative biotransformations must be performed. However, in some cases, such as oxygenase catalyzed reactions, it is a real challenge to transfer the biotransformations from the analytical scale to the semi-preparative scale. Oxygenases incorporate one or two atoms of molecular oxygen in a multitude of organic compounds, but are often limited by low stability, cofactor dependency, substrate/product inhibition and in particular low oxygen mass transfer [2–4]. Some of these limitations can be overcome by the employment of whole cell catalysis instead of isolated enzyme [2]. Nevertheless, oxygen limitations remain a true challenge in oxygenase catalyzed reactions. To date, various strategies were used to reduce oxygen limitation in biotransformations, such as use of oxygen-enriched air [5, 6], increased interfacial surface area [7], strong stirring or increased oxygen partial pressure [8].

Rieske non-heme iron oxygenases (ROs), for instance, belong to the class of oxygenases and catalyze the regio-, stereo- and enantioselective hydroxylation of a wide range of organic compounds, ranging from aromatic to aliphatic compounds and monoterpenes [9–14]. In addition, ROs exhibit a broad reaction scope, covering *cis*-dihydroxylations, monohydroxylations, sulfoxidations, desaturations, epoxidations and *O*- and *N*-dealkylations [10, 11, 15–17], making these multicomponent systems promising for diverse industrial applications.

Here, we compared different RO scale-up strategies used in literature and further enhanced the most promising approach for the *cis*-hydroxylation of naphthalene **1** to the so far unreported (1*R*, 2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol **2** catalyzed by the cumene dioxygenase wild type from

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Pseudomonas fluorescens IP01 (CDO). Further, we employed the most beneficial working conditions for the monohydroxylation of (*R*)-(+)-limonene to (+)-*trans*-carveol with the previously described CDO variant A283del [14]. With our setup, semi-preparative biotransformation can be performed fast and easy with equipment available in most chemical laboratories.

2 Materials and Methods

A detailed description of the materials and methods, the recombinant bacterial strain, growth and expression conditions, biotransformation as well as analysis methods via HPLC-DAD and product characterization via ^{1}H- and ^{13}C-NMR can be found in the Supporting Information (Sect. S1 and S2).

3 Results and Discussion

3.1 Study of Scale-Up Conditions

In the literature, semi-preparative biotransformations of ROs have been carried out in orbital shakers in flasks, baffled flasks or on magnetic stirrers in round bottom flasks [11, 13, 18–21]. However, to the best of our knowledge no comparison of different semi-preparative biotransformation setups for ROs has been performed so far. Thereby, oxygen limitation is a major problem in RO biotransformations [2–4]. In order to find the best conditions for semi-preparative biotransformations with resting cells expressing ROs, the dihydroxylation of **1** to **2**, catalyzed by recombinantly produced CDO wild type, was studied (Fig. 1a). All reactions were done with 10 mM **1** in a total volume of 100 mL and were analyzed by HPLC-DAD. In order to guarantee the comparability of our results, the experiments were performed with homogeneous cells from the same expression. Thus, differences in expression cannot affect the results. To

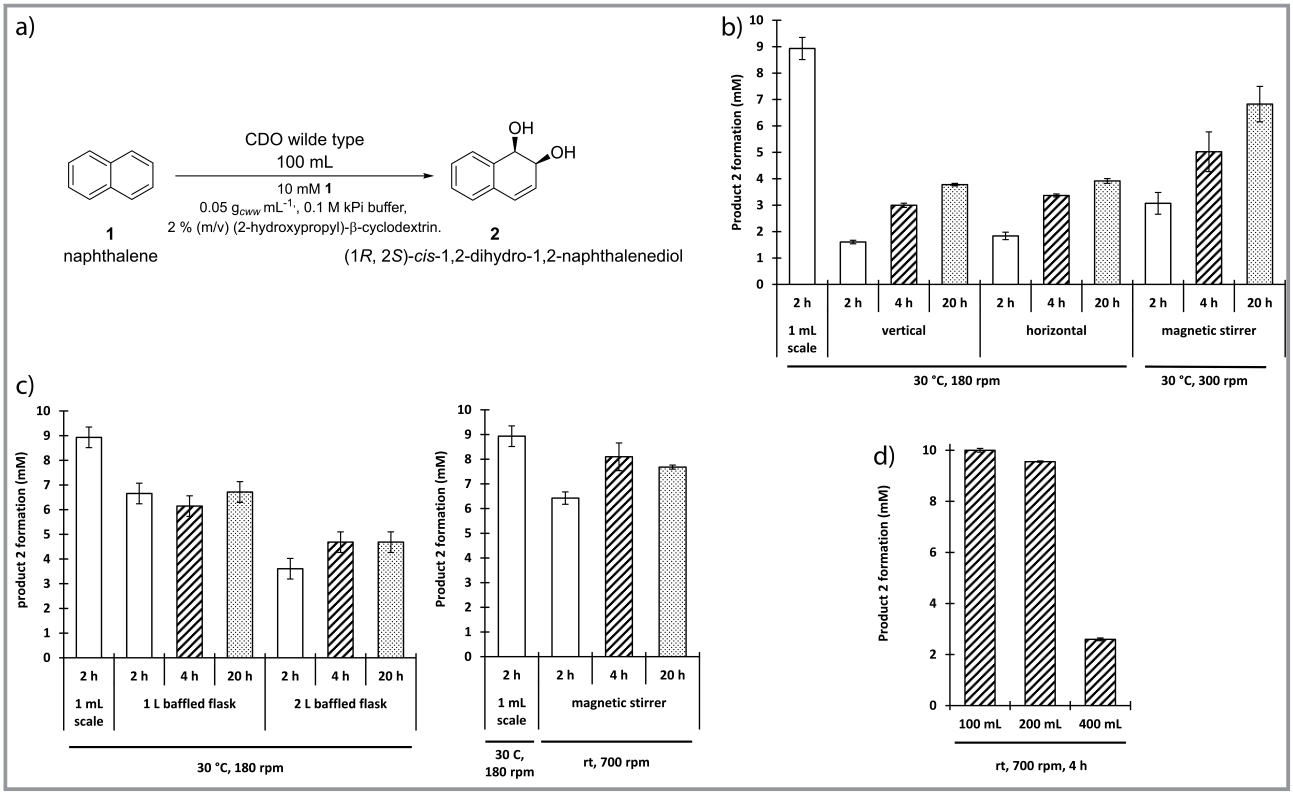

Figure 1. Study of semi-preparative scale-up conditions of CDO wild type biotransformations. Reactions were performed in 100 mL total volume with 10 mM **1**, 0.05 $g_{cww}mL^{-1}$ resting cells if not stated otherwise. Samples were taken in technical triplicates and analyzed by HPLC-DAD. To ensure comparability of the conditions tested, all semi-preparative biotransformations shown in one diagram were performed with homogeneous cells from the same expression. a) Dihydroxylation of **1** to **2** catalyzed by recombinant produced CDO wild type, used as a model reaction for the enhancement of the semi-preparative biotransformation. b) Comparison of the product **2** formation between 2 L Schott bottles incubated vertical and horizontal at 30 °C, 180 rpm in an orbital shaker and on a magnetic stirrer at 300 rpm. The results of the semi-preparative biotransformations are compared to the 1 mL scale. c) Comparison of the product **2** formation between 1 L and 2 L baffled flasks incubated at 30 °C, 180 rpm in an orbital shaker and a 2 L round bottom flask incubated at rt, 700 rpm on a magnetic stirrer. The results of the semi-preparative biotransformations are compared to the 1 mL scale. d) Product **2** formation of the semi-preparative biotransformations of **1** stirred in a 2 L round bottom flask with either 100 mL, 200 mL or 400 mL reaction volume at 700 rpm, rt for 4 h.

overcome oxygen limitation, agitation strategies were investigated in 2 L Schott bottles with an air to liquid ratio of 19:1, to ensure sufficient oxygen in the headspace (Fig. 1b). Hence, semi-preparative biotransformations were performed at 30 °C in 2 L Schott bottles and were incubated horizontally or vertically in a shaker at 180 rpm or on a magnetic stirrer at 300 rpm (Fig. 1b). In all three cases, product **2** formation increased over time, with the increase being greatest for the magnetic stirrer. Magnetic stirring leads to ca. 1.8-fold higher product **2** formation (6.83 ± 0.67 mM) after 20 h compared to vertical (3.78 ± 0.05 mM) and horizontal (3.92 ± 0.09 mM) incubation. Vertical or horizontal incubation exhibited only minor differences. This could be attributed to a better mixing with the magnetic stirrer and therefore a better oxygen transfer into the aqueous phase. Such a positive influence of vigorous stirring was already shown for Baeyer-Villiger monooxygenases in continuous stirred tank reactors [6].

To further increase the oxygen transfer in the semi-preparative biotransformation, we compared 1 L and 2 L baffled flasks in an orbital shaker at 180 rpm and 30 °C, with a 2 L round bottom flask on a magnetic stirrer at 700 rpm and room temperature (rt) (Fig. 1c). Regarding the baffled flasks, the 1 L flask showed higher product **2** formations than the 2 L flask. This can be explained by the higher liquid level in the 1 L flask, leading to a larger contact area between the liquid and the baffles and thus to better mixing and oxygen transfer into the reaction medium. After 2 h, the incubation in 1 L baffled flask and the 2 L round bottom flask showed the highest product **2** formation with 6.66 ± 0.30 mM and 6.42 ± 0.25 mM. In contrast to the 1 L baffled flask, the product **2** formation of the stirred biotransformation in a 2 L round bottom flask at 700 rpm further increased to 8.10 ± 0.56 mM after 4 h. Overall, we found that the best agitation strategy for CDO up-scaling was stirring of 100 mL reaction mixture at 700 rpm, on a magnetic stirrer at rt. It is worth mentioning that with the enhanced setup, it is possible to achieve similar product **2** formations as in the 1 mL scale (8.93 ± 0.42 mM). During the semi-preparative biotransformation, we observed a strong foaming. To determine if foaming affects product formation, semi-preparative biotransformations with our enhanced setup were performed without and with 0.005 % of the antifoam agent Antifoam 204. However, use of Antifoam 204 lead to ca. 40 % decrease in product **2** formation (Fig. S1), which might be explained by the larger gas to liquid volume ratio, caused by the foam [22]. Moreover, antifoam agents reduce the oxygen mass transfer into the aqueous reaction medium. [22]

Next, we wanted to evaluate our enhanced semi-preparative biotransformation setup by isolating the product **2**. After extraction and purification 130 mg of **2** were obtained with a yield of 80 % (1.3 g L^{-1}) and the enantioselectivity was determined for the first time, showing that the (1*R*, 2*S*) enantiomer of **2** is formed with > 99 % *ee* (Fig. S7). This is the same enantiomer that is also formed by the wild types of toluene dioxygenase and naphthalene dioxygenase [10, 23].

To investigate the influence of the reaction volume on the semi-preparative biotransformation, reactions were performed in 100 mL, 200 mL and 400 mL, using the enhanced parameters (Fig. 1d). Thereby, almost full conversion was achieved with 100 mL and 200 mL reaction volume. The differences in product **2** formation of 100 mL biotransformation compared to Fig 1c results from variations in gene expression. Interestingly, an enlargement to 400 mL leads to a 3.8-fold decrease in product **2** formation after 4 h compared to 100 mL. These results suggest that oxygen supply is insufficient in the 400 mL setup, possibly caused by the smaller headspace in the reaction vessel or by insufficient mixing. Thus, with our enhanced setup, reactions with 200 mL reaction volume can be performed to further increase the amount of isolated product.

3.2 Air vs Oxygen Headspace

A commonly used technique to increase oxygen transfer in aqueous media, is to use oxygen enriched air or to increase air flow rates in the reactor [5, 6, 8]. Therefore, we wanted to check if an oxygen-enriched headspace could further increase the product **2** formation. Thus, the influence of an oxygen-enriched headspace in the 2 L round bottom flask was compared to normal air headspace. The enhanced setup was used and a time course of the CDO catalyzed reaction of **1** to **2** was recorded for air headspace and oxygen headspace with 100 mL reaction mixture over 1 h (Fig. 2). Interestingly, the use of oxygen-enriched headspace leads to ca. 2.8-fold increase in product **2** formation after 0.5 h compared to air headspace, indicating once again that oxygen supply is a limiting factor for this reaction. Furthermore, using oxygen-enriched headspace, full conversion was achieved after 1 h.

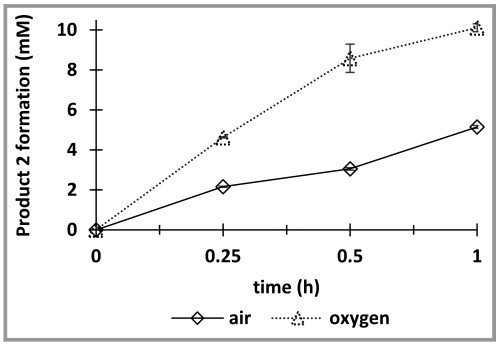

Figure 2. Time course of the product **2** formation with the enhanced setup with air (solid line) and oxygen-enriched headspace (dashed line). Reactions were incubated on a magnetic stirrer in a 2 L round bottom flask at 700 rpm and rt in a total volume of 100 mL with 10 mM **1**.

3.3 Semi-Preparative Biotransformation of (*R*)-(+)-Limonene

Furthermore, we wanted to show the usefulness of our enhanced semi-preparative setup for CDO catalyzed conversion of the volatile monoterpene (*R*)-(+)-limonene **3** to the valuable compound (+)-*trans*-carveol **4** (Fig. 3). To further increase product formation, the previously described CDO variant A283del [14] was employed. The reaction was performed in 200 mL total volume with 10 mM **3** under oxygen-enriched headspace for 4 h. After extraction and purification by column chromatography, **4** was obtained with 78.9 % yield (240 mg), showing the potential of our approach for other substrates. In contrast to other approaches, which bubble air through the reaction [6] or use flasks for incubation [21], our setup in the round bottom flask can be properly sealed and is therefore suitable for volatile compounds. It is also worth mentioning that **4** can be produced with a titer of 1.2 g L^{-1} and a higher isolated yield than previously reported for biocatalytic production of **4** [24].

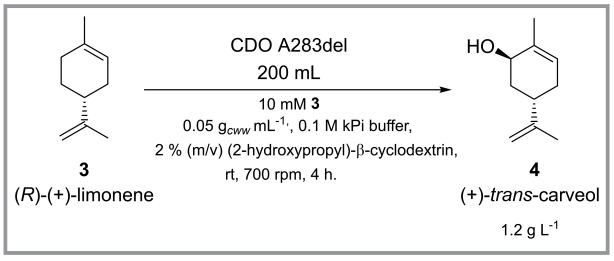

Figure 3. Semi-preparative biotransformation of **3** to **4** catalyzed by recombinant produced CDO variant A283del. The reaction was performed with 10 mM **3**, 0.05 $g_{cww}mL^{-1}$ resting cells, using a total volume of 200 mL in a 2 L round bottom flask, stirred at 700 rpm on a magnetic stirrer at rt for 4 h under oxygen-headspace.

4 Conclusion

In this work, we enhanced the semi-preparative biotransformation of **1** and **3** catalyzed by CDO. The most advantageous setup for the semi-preparative reaction was found to be incubation at rt on a magnetic stirrer in a 2 L round bottom flask at 700 rpm, in a total volume of 200 mL under oxygen-enriched headspace. The experiments demonstrate a useful setup for the biosynthesis of the first hundred milligrams of product, enough for NMR analysis and characterization of unknown products, even for volatile compounds. Our enhanced semi-preparative scale-up can be performed with equipment available in most chemical laboratories and no bioreactor is required. We assume that this established setup can also be expanded for other ROs and oxygenases with only minimal adaptions, which is a promising opportunity for lab-scale product identification.

Supporting Information

Supporting Information for this article can be found under DOI: https://doi.org/10.1002/cite.202200162. This section includes additional references to primary literature relevant for this research [25, 26].

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Abbreviations

CDO	cumene dioxygenase from *Pseudomonas fluorescens* IP01
cww	cell wet weight
DAD	diode array detector
GC	gas chromatography
HPLC	high performance liquid chromatography
kP_i	potassium phosphate
NMR	nuclear magnetic resonance
ROs	Rieske non-heme iron oxygenases
rpm	revolutions per minute
rt	room temperature

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