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Towards preparative peroxygenase-catalyzed oxyfunctionalization reactions in organic media



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ABSTRACT

The peroxygenase from *Agrocybe aegerita* (AaeUPO) has been evaluated for stereoselective oxyfunctionalization chemistry under non-aqueous reaction conditions.

The stereoselective hydroxylation of ethylbenzene to (*R*)-1-phenylethanol was performed in neat substrate as reaction medium together with the immobilized biocatalyst and ^{tert}BuOOH as oxidant.

Stability and activity issues still have to be addressed. Nevertheless, gram-scale production of enantiopure (*R*)-1-phenylethanol was achieved with respectable 90,000 turnovers of the biocatalyst.

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1. Introduction

Peroxygenases catalyze a broad range of synthetically interesting oxyfunctionalization reactions. [1,2] Amongst them, stereospecific hydroxylation of alkyl benzenes is worth mentioning as chemical catalysts with comparable selectivity and activity are still missing [3]. Furthermore, peroxygenases excel over the well-known P450 monooxygenases by their simplicity needing simple hydrogen peroxide or organic hydroperoxides as cosubstrates instead of the nicotinamide cofactor and complicated electron transport chains [4,5].

The chloroperoxidase from *Caldariomyces fumago* (CfUPO) represents the first example of an ‘unspecific’ peroxygenase (E.C. 1.11.2.1) exhibiting significant P450-like activity (e.g. C–H-bond activation) [6,7]; and major research efforts had been devoted to the exploration of its properties, product spectrum and possible applications. Unfortunately, however, CfUPO’s catalytic activity towards non-activated or poorly activated C–H bonds is comparably low impairing its preparative usefulness. In 2004 the group

around Hofrichter reported another peroxygenase from the fungus *Agrocybe aegerita* (AaeUPO) exhibiting significantly higher activity [8]. Today, more than 300 substrates have been reported for AaeUPO that often are converted highly chemo- and enantioselectively [1,2]. Furthermore, recombinant expression systems for AaeUPO are available [9] enabling protein engineering [10,11]. Also a crystal structure of AaeUPO is known facilitating (semi-)rational protein engineering [12].

Overall, AaeUPO is an extremely promising candidate biocatalyst for preparative-scale, selective oxyfunctionalization chemistry.

One major limitation of AaeUPO (and of peroxygenase-catalysis in general) however still is its limitation to aqueous reaction conditions, which poses a major challenge to the conversion of poorly water soluble, hydrophobic starting materials such as alkyl benzenes. Both, from an economical and an environmental point of view, higher substrate loadings than traditionally used are highly desirable [13–15]. The use of cosolvents to increase the water solubility of the hydrophobic starting materials or two-liquid-phase approaches using a hydrophobic cosolvent as substrate reservoir and product sink have been proposed [16–19]. Avoiding additional solvents at all and performing the transformations in neat conditions (i.e. without any cosolvent whatsoever) would be the most elegant methodology.

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Therefore, in the present study, we set out to evaluate the feasibility of peroxxygenase-catalyzed oxyfunctionalization reactions under non-aqueous conditions. As the model enzyme we chose *AaeUPO*, recombinantly expressed in *Pichia pastoris* (*rAaeUPO*) [9], the model reaction was the stereoselective hydroxylation of ethylbenzene to (*R*)-1-phenylethanol.

2. Materials

2.1. Chemicals and enzymes

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) in the highest purity available and used without further purification except ethylbenzene that was freshly distilled prior use to remove the traces of phenylethanol and acetophenone. The enzyme carrier ReliZyme™ HA403/M, a macroporous PMMA resin with amino-functionalizations, was obtained from Resindion S.r.l., Italy.

2.2. Enzyme production

The recombinant peroxxygenase from the basidiomycetous fungus *Agrocybe aegerita* (*rAaeUPO*) was produced via heterologous fermentation in *Pichia pastoris* following a previously described procedure [9].

2.3. Concentration of *rAaeUPO*

The concentration of *rAaeUPO* was determined using the molar extinction coefficient of $115 \text{ mM}^{-1} \text{ cm}^{-1}$ at 420 nm. Absorption spectra in the UV/vis range was recorded in a Biomate5 (Thermo) spectrophotometer (Fig. 1). The Reinheitszahl (Rz value) is the ratio of absorbance due to hemin (A420, Soret region) to absorbance due to protein (A280) and therewith a measure for the protein purity. The Rz-value of the current *rAaeUPO* preparation was 1.6 corresponding well to values reported in the literature [9–11].

2.4. Immobilization of *rAaeUPO* on ReliZyme™ HA403/M resin

To immobilize the peroxxygenase the following procedure was used: ReliZyme™ HA 403/M resin (1 g) was treated with 50 mL of 0.125% glutaraldehyde solution in water for 2.5 h in a shaking device at 16 °C. The glutaraldehyde solution was then removed by centrifugation, and the resin was washed three times with 0.1 M phosphate buffer at pH 7. The buffer was then removed, and 3 mL of pure *rAaeUPO* (1.35 mg) and 1 mL of 0.1 M phosphate buffer at pH 7 were added to the activated support. The mixture was incubated in a shaker for 24 h at 16 °C. The residual enzymatic activity in the solution was monitored by using the ABTS oxidation assay (*vide infra* for details). The resin was then washed with 50 mM phosphate buffer at pH 7, dried and stored at 4 °C.

2.5. *rAaeUPO* concentration in the beads

The amount of the *rAaeUPO* bound to the resin was determined by subtracting the amount of enzyme present in the supernatant after immobilization from the total amount of enzyme present originally (prior addition of the resin). The measurement was done spectrophotometrically (Fig. 1) using the molar extinction coefficient of $115 \text{ mM}^{-1} \text{ cm}^{-1}$ at 420 nm. Quite reproducibly, 1.35 mg *rAaeUPO* per gram of resin was bound (i.e. quantitative immobilization).

2.6. Activity determination of *rAaeUPO*

In order to quantify the specific activity of *rAaeUPO*, we used ABTS as a substrate in aqueous media. Absorbance changes during ABTS oxidation in 0.1 M citrate buffer pH 5 were recorded at 25 °C in a Biomate5 (Thermo) spectrophotometer. The reactions were initiated by the addition of 5 mM H_2O_2 . Oxidation of ABTS was followed by the formation of the cation radical ($\epsilon_{405} 36.8 \text{ mM}^{-1} \text{ cm}^{-1}$). Different concentrations of *rAaeUPO* were used to create a calibration line based on the activity towards ABTS (0.5 mM ABTS in 0.1 M citrate buffer and 5 mM H_2O_2).

The activity of the immobilized enzyme was estimated in a reaction mixture of 5 mL containing 7 mg beads, 0.5 mM ABTS and 5 mM H_2O_2 in citrate buffer (pH 5.0) magnetically stirred at room temperature. Aliquots were withdrawn every 30 s and measured at 405 nm. Reactions were performed in duplicates.

2.7. Hydroxylation of ethylbenzene

Reactions were performed at 30 °C and ambient atmosphere in 1 mL ethylbenzene containing different amount of immobilized *rAaeUPO*. Every 30 min tertBuOOH was added into the reaction mixture and samples were collected. Samples were mixed with ethyl acetate (containing 5 mM 1-octanol as internal standard) and analysed by GC. Reactions were performed in duplicates.

2.8. Stability of immobilized *rAaeUPO* in ethylbenzene

The immobilized *rAaeUPO* was incubated in ethylbenzene at 30 °C for 24 h and the residual activity was measured following ethylbenzene hydroxylation after the addition of 10 mM tertBuOOH and 1 h of incubation.

2.9. Stability of immobilized *rAaeUPO* against peroxide

The immobilized *rAaeUPO* (7 mg) was incubated in phosphate buffer (pH 5.0) containing 10 mM tertBuOOH and the residual activity towards ABTS was measured as described above.

2.10. Immobilization of *PpAOx* on ReliZyme™ HA403/M resin

The ReliZyme™ HA 403/M resin (1 g) was treated with 50 mL of 0.125% glutaraldehyde solution in water for 2.5 h in a shaking device at 18 °C. The glutaraldehyde solution was then removed by centrifugation, and the resin was washed three times with 0.1 M phosphate buffer at pH 7. The buffer was then removed, and 9 mL of *PpAOx* (15 mg) and 1 mL of 0.1 M phosphate buffer at pH 7 were added to the activated support. The mixture was incubated in a shaker for 24 h at 18 °C: the residual enzymatic activity in the solution was assayed by ABTS oxidation assay (0.1 M phosphate pH 7, 0.033% methanol, 2 mM ABTS and 2.5U HRP) described by Sigma Aldrich. The resin was then washed with 50 mM phosphate buffer at pH 7, dried and stored at 4 °C.

2.11. Concentration of *PpAOx* on the beads

The amount of the enzyme added for the immobilization and the enzyme retained in the supernatant was quantify with the BSA assay. Based on this assay, 15 mg of protein was bound per mg of beads (i.e. quantitative immobilization).

2.12. Activity of *PpAOx*

In order to quantify the specific activity of the immobilized *PpAOx* we used the assay recommended by the supplier (Sigma Aldrich): the reaction conditions were: 0.1 M phosphate

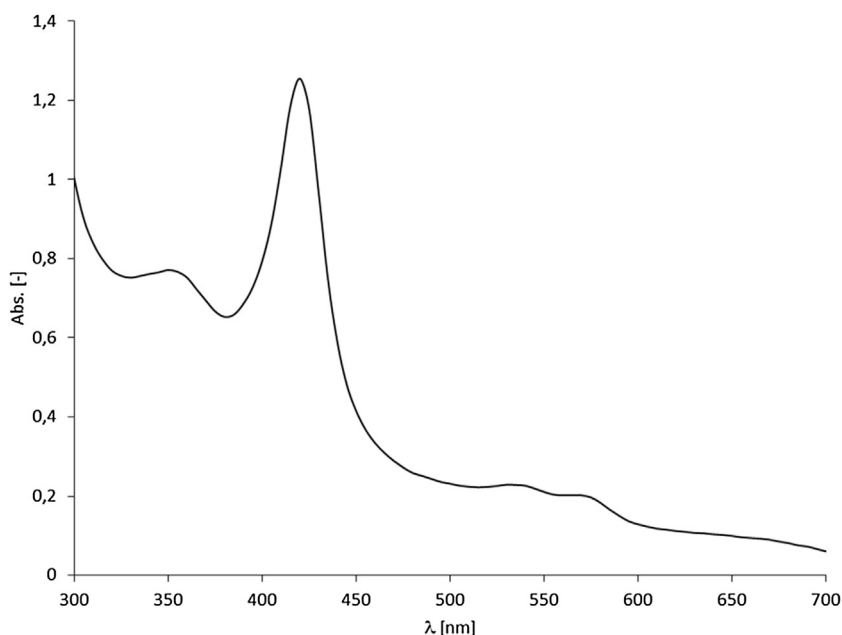


Fig. 1. UV/vis spectrum of purified rAaeUPO.

pH 7, 0.033% methanol, 2 mM ABTS and 2.5U HRP. Oxidation of ABTS was followed by the formation of the cation radical (ϵ_{405} $36.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.13. Reactions in two-liquid phase system (2LPS)

The two-liquid-phase system consisted of ethylbenzene and phosphate buffer (pH 7.0) (phase ratio = 1:1) containing aqueous concentrations of $[\text{rAaeUPO}] = 100 \text{ nM}$, $[\text{PpAOX}] = 60 \text{ nM}$, $[\text{FDM}] = 0.2 \text{ gL}^{-1}$ and 200 mM methanol. Aliquots were withdrawn from clearly separated organic phase at interval, mixed with equal volume of ethyl acetate (containing 5 mM dodecane) and analysed by GC. Reactions were performed in duplicates.

3. Methods

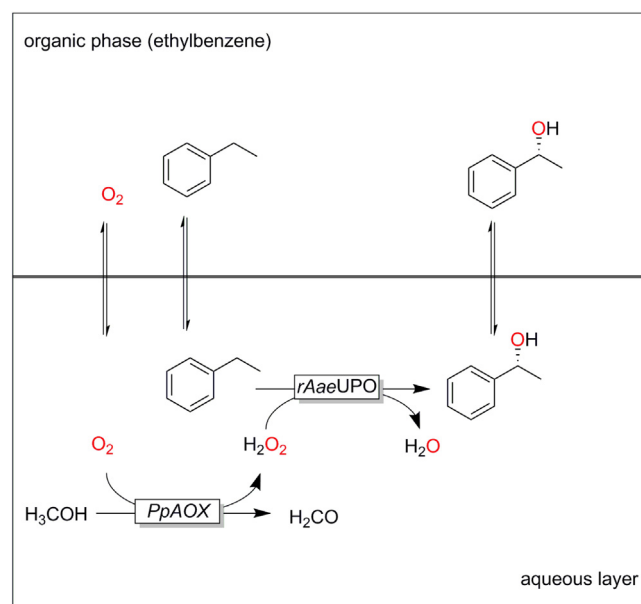
3.1. GC analytics

At intervals, samples were withdrawn from the reaction mixtures and extracted three times with the same volume of ethyl acetate (containing 5 mM octanol or 5 mM dodecane as internal standard). The combined organic phase was dried with magnesium sulfate and centrifuged. The supernatant was analysed by GC (see Fig. 2 for an exemplary chromatogram with authentic standards). Concentrations reported have been determined based on calibration curves using authentic standards.

4. Results and discussion

The starting point of our investigation was a two-liquid phase system using ethylbenzene as substrate reservoir and product sink. For *in situ* H_2O_2 generation we chose the previously described alcohol oxidase-catalyzed oxidation of methanol (Scheme 1).

As shown in Fig. 3, reactions employing diffusible enzymes resulted in comparably fast product formation (2.9 mM h^{-1} , $\text{TF}(\text{rAaeUPO}) = 8.1 \text{ s}^{-1}$, $\text{TF}(\text{PpAOx}) = 13.4 \text{ s}^{-1}$). We hypothesize that the overall productivity of the reaction system may have been limited by oxygen transfer into the aqueous reaction medium. In a previous study using a comparable setup a volumetric productivity around 2.5 mM h^{-1} had been observed, which was attributed to



Scheme 1. Enantioselective hydroxylation of ethylbenzene using the two liquid phase (2LP) approach and methanol oxidation for *in situ* H_2O_2 generation. Ethylbenzene served as organic phase (substrate reservoir and product sink).

diffusion limitation over the interphase [20]. Also the robustness of this preliminary setup was comparably poor with a complete loss of ethylbenzene hydroxylation activity after 24 h. At present stage we have no further insight into the molecular reason for this poor robustness. Possibly, the mechanically demanding conditions (such as shear stress or the presence of a hydrophobic interphase) account for this low long-term stability and further in-depth investigations are necessary to clarify this issue. Nevertheless, rAaeUPO and PpAOx performed 206,000 and 343,000 catalytic turnovers, respectively.

To address the poor stability of the biocatalysts under the reaction conditions we evaluated using immobilized enzyme preparations. As shown in Fig. 3, the reaction utilizing immobilized rAaeUPO and PpAOx was significantly more robust but also significantly less active. Using the same nominal enzyme concen-

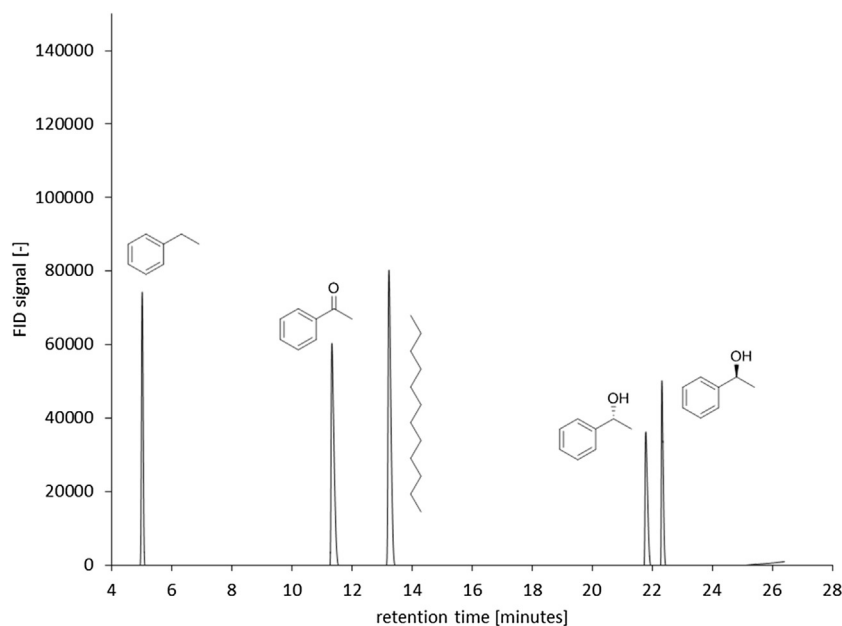


Fig. 2. Exemplary GC chromatogram of authentic standards. Temperature profile: 90 °C for 5 min, 20 °C/min to 110 for 15 min, 25 °C/min to 220 for 1 min. Peak assignment: ethylbenzene (5.0 min), acetophenone (11.3 min), dodecane (13.2 min), (R)-1-phenylethanol (21.8 min), (S)-1-phenylethanol (22.3 min).

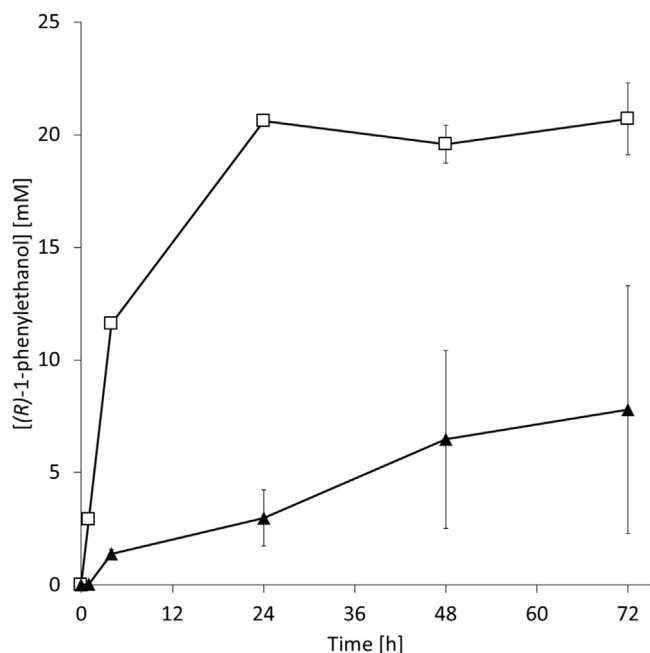
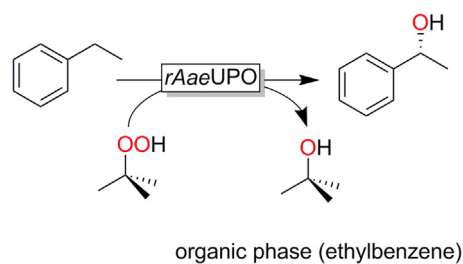


Fig. 3. rAaeUPO-catalyzed hydroxylation of ethylbenzene in a two-liquid-phase system (2LPS) using free rAaeUPO and free PpAOx (□) or immobilized rAaeUPO and PpAOx (▲). Conditions: 2LPS ($V_{\text{ethylbenzene}} = V_{\text{aq}} = 1$ mL), 100 mM phosphate buffer (pH 7.0), [methanol] = 200 mM, [rAaeUPO] = 100 nM, [PpAOx] = 60 nM, $T = 30$ °C. In both cases, (R)-1-phenylethanol was the major product (>95%, ee > 98%) with less than 5% of the overoxidation product (acetophenone, left out for reasons of clarity).

trations as compared to the free enzymes, the product formation rate dropped to 0.11 mM h^{-1} concomitantly leading to a reduction of the enzymes' performance by more than 20-fold. This is in line with the finding that the immobilized enzymes are significantly less active than their free pendants.

Having the immobilized enzymes at hand, we drew our attention to their use under neat conditions (i.e. using ethylbenzene as sole reaction medium; in other words the aqueous layer of Scheme 1 is reduced to a minimum originating from the im-



Scheme 2. Enantioselective hydroxylation of neat ethylbenzene using immobilized rAaeUPO and tertBuOOH as oxidant.

bilization procedure). In a first set of experiments we evaluated the bi-enzymatic cascade using PpAOx for *in situ* H_2O_2 generation. However, only trace amounts of product were detectable under these reaction conditions. Possibly, the *in situ* formed H_2O_2 (and/or formaldehyde) accumulated quickly in the small aqueous layer to critical concentrations and lead to fast enzyme inactivation. Therefore, we decided to use tertBuOOH as peroxide donor to promote the reaction (Scheme 2).

tertBuOOH proved to be a milder oxidant as compared to H_2O_2 as the oxidative inactivation of the rAaeUPO heme was significantly slower (Fig. 4).

The stability of the immobilized enzyme in pure ethylbenzene was found to be acceptable with approximately 46% activity loss over 24 h (Fig. 5).

The dosage of tertBuOOH had a very significant effect on the rate and robustness of the hydroxylation reaction (Fig. 6). The overall reaction rate almost linearly correlated with the amount of hydro peroxide added while the robustness of the reaction followed an opposite trend: adding tertBuOOH at 40 mM h^{-1} lead to a complete cease of product formation after 30 min at latest. However, lower dosing rates of 10 or 20 mM h^{-1} resulted in fairly linear product accumulation over the reaction time observed. Most likely this behavior can be explained by the increasing enzyme activity with increasing availability of the cosubstrate (tertBuOOH) and the increasing oxidative inactivation of the prosthetic heme-group with increasing peroxide concentrations.

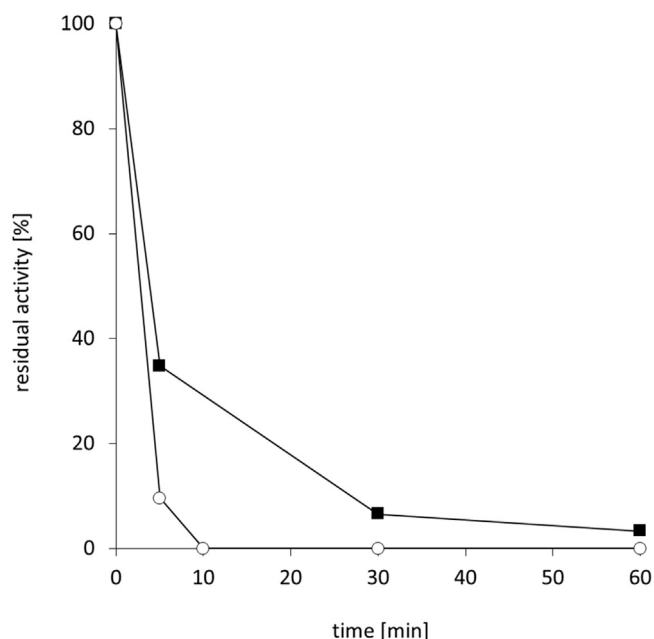


Fig. 4. Stability of immobilized rAaeUPO against peroxide. The immobilized rAaeUPO was incubated in phosphate buffer (pH 5.0) containing 10 mM tertBuOOH (■) and 10 mM H_2O_2 (○), at intervals samples were withdrawn and subjected to an activity assay. Error bars are not shown since the standard deviation was always below 5%.

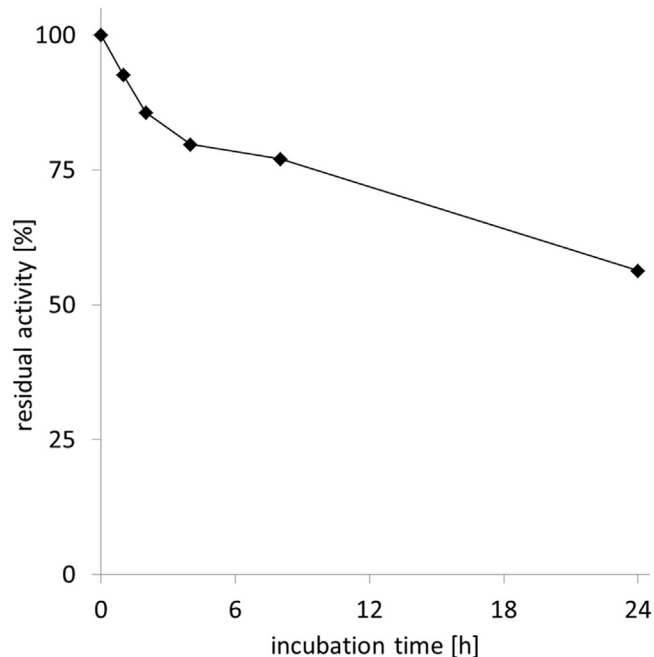


Fig. 5. Stability of immobilized rAaeUPO suspended in neat ethylbenzene. The residual activity was determined upon addition of 10 mM of tertBuOOH and incubation of 1 h at 30°C . Conditions: ethylbenzene and $[\text{rAaeUPO}] = 400 \text{ nM}$ at 30°C . Error bars are not shown since the standard deviation was always below 5%.

In all reactions a certain amount of overoxidation of (*R*)-1-phenylethanol to acetophenone was observed culminating in 13–21 mM of this undesired side product. Here it is interesting to note that this overoxidation was significant in the first phase of the reaction while after approx. 30–60 min this reaction slowed down significantly leading to a relatively stable level of acetophenone. Today, we are lacking a plausible explanation for this observation.

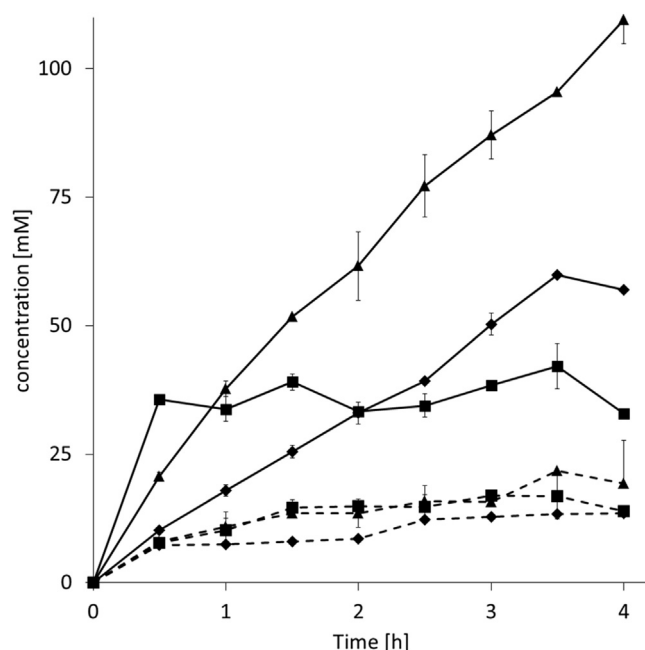


Fig. 6. Comparison of the effect of different tertBuOOH dosing rates on the productivity and robustness of the rAaeUPO-catalyzed hydroxylation of ethylbenzene. General conditions: ethylbenzene was used as sole liquid phase supplemented with immobilized rAaeUPO (corresponding to an overall concentration of $35 \mu\text{M}$), $T = 30^\circ\text{C}$, tertBuOOH was added portion wise at 30 min intervals corresponding to ■: 40 mMh^{-1} , ▲: 20 mMh^{-1} , ◆: 10 mMh^{-1} ; straight lines correspond to (*R*)-1-phenylethanol, dotted lines correspond to acetophenone.

Possibly, the accumulating tertBuOH inhibited the overoxidation; further investigations are currently underway.

It should be mentioned that in these experiments comparably large nominal rAaeUPO concentrations ($35 \mu\text{M}$) had been employed to compensate for the poor activity of the immobilized enzyme. Hence, the catalytic performance of rAaeUPO was rather poor (2700 catalytic turnovers in 3 h). Further experiments with significantly reduced enzyme concentrations (400 nM and 600 nM , respectively) were conducted (Fig. 7) revealing the importance of the enzyme amount on the robustness of the overall reaction: While using large amounts ($35 \mu\text{M}$) of enzyme and a nominal tertBuOOH dosing rate of 20 mMh^{-1} linear product accumulation was observed throughout the experiments. However, using low (400 nM or 600 nM) concentrations of rAaeUPO product accumulation ceased after 1.5 h and 2.5 h, respectively. Nevertheless, up to 40 mM of enantiopure (*R*)-1-phenylethanol was formed under these conditions corresponding to a total turnover number of 67,500 for the enzyme.

Overall we conclude that the ratio of tertBuOOH addition to enzyme concentration is the decisive factor determining the efficiency of the overall reaction in terms of volumetric productivity and total turnover of the enzyme. Further optimization studies are currently ongoing in our laboratory. Particularly, controlling the water activity is being investigated to optimize the enzyme activity and optimization of the enzyme loading on the carrier are currently ongoing.

Despite the comparably early stage of development, we proceeded to semi-preparative scale to demonstrate the practical feasibility of the proposed reaction setup. On 250 mL scale a total amount of 1.25 g of (*R*)-phenylethanol (isolated yield) were obtained within 3 h of reaction time yielding a respectable TTN(rAaeUPO) of more than 90,000. Hence, we are convinced that an optimized reaction scheme exhibits significant potential for synthetic organic chemistry.

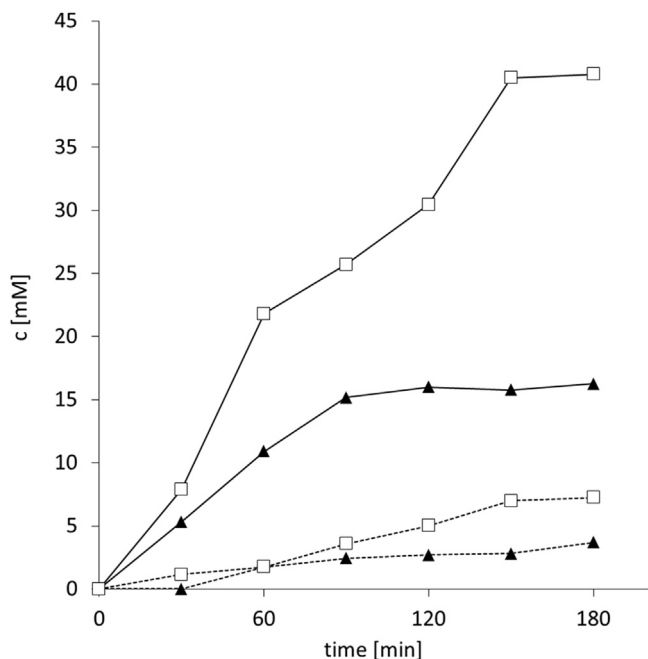


Fig. 7. Comparison of the effect of different concentrations of immobilized rAaeUPO (400 nM $\blacktriangle$, 600 nM $\square$), 20 mMh⁻¹ of *tert*-BuOOH was added at 30 min intervals to promote rAaeUPO catalyzed hydroxylation of ethylbenzene. (R)-1-phenylethanol: solid lines, acetophenone: dashed lines. Conditions: ethylbenzene and [rAaeUPO] = 400 or 600 nM at 30 °C. Reaction volume 250 mL. Error bars are not shown since the standard deviation was always below 5%.

5. Conclusions

Today, comparably few studies deal with the application of oxidoreductases under non-aqueous conditions. The poor aqueous solubility of most reagents of interest however necessitates intensified research on biocatalysis in 'neoteric' solvents i.e. alternative solvents enabling higher substrate payloads. For synthetic applications this is of utmost importance as chemists will accept and apply only those methods that give them access to practical product amounts.

In the present study we have taken the first steps towards using peroxygenases under non aqueous reaction conditions. Using

immobilized rAaeUPO suspended in the neat starting material (ethylbenzene) we could provide the first evidence for this reaction setup. The limitations identified so far comprise (1) the low residual activity of the heterogenized biocatalyst and (2) the comparably poor stability of the enzyme under non-aqueous conditions. Future studies will have to address these shortcomings. Nevertheless, despite the very early stage of development of this system, preparative, gram-scale synthesis of enantiopure (R)-1-phenylethanol could be demonstrated, which makes us confident that an optimized reaction system may exhibit some preparative relevance.

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