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DOI

[10.1016/j.mcat.2023.113396](https://doi.org/10.1016/j.mcat.2023.113396)

Publication date

2023

Document Version

Final published version

Published in

Molecular Catalysis

Citation (APA)

Wu, B., Wang, X., Yang, B., Ma, Y., Hollmann, F., & Wang, Y. (2023). Biosynthesis of cyclic ketones by a H₂O₂ self-sufficient cascade reaction. *Molecular Catalysis*, 547, Article 113396. <https://doi.org/10.1016/j.mcat.2023.113396>

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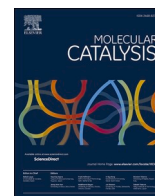
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Biosynthesis of cyclic ketones by a H₂O₂ self-sufficient cascade reaction

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ARTICLE INFO

Keywords:

Cyclohexanone
Alcohol oxidase
Unspecific peroxygenase
H₂O₂ self-cycling

ABSTRACT

In the present work we propose a bienzymatic cascade for the oxyfunctionalisation of cycloalkanes to cyclic alcohols/cyclic ketones. By combining a H₂O₂-dependent peroxygenase with a O₂-consuming and H₂O₂-producing alcohol oxidase an overall aerobic oxidation system was established. A convincing proof-of-concept is presented and some current limitations are outlined.

Introduction

The oxidation of cyclohexane to cyclohexanol and cyclohexanone represents an essential step in the production of caprolactam and adipic acid, intermediates in the manufacture of nylons [1]. This transformation is performed at large industrial scale by various companies using catalytic aerobic oxidation. The reaction products, however, are significantly more reactive than the hydrocarbon starting material, leading to the formation of considerable amounts of undesired by-products. Therefore, industrial transformations of cyclohexane are generally run until not more than 5% conversion to minimise the undesired side-reactions [1].

Enzymes are alternative catalysts e.g. for the hydroxylation of cyclohexane, holding the promise of more selective transformations [2–3]. Indeed, biocatalytic oxidations of cyclohexane to cyclohexanol have been reported using so-called P450 monooxygenases. Enzyme stability issues as well as their dependency on nicotinamide cofactors and complex electron transport chains limit their practical applicability [4–9]. The product titres of these monooxygenase-based reaction systems tend to be low (in the lower millimolar range). Partially, this can be attributed to the complex molecular architecture of monooxygenases [10]. Preparative industrial applications of such systems remain highly challenging.

Recently, peroxygenases have emerged as robust alternatives to existing P450 monooxygenases to catalyse the challenging hydroxylation of non-activated alkanes such as cyclohexane [11–13]. Very promisingly, using the peroxygenase from *Agroclybe aegerita* (*AaeUPO*),

highly chemoselective transformation of cyclohexane into cyclohexanol was observed with only cyclohexanone as sole by-product even at high cyclohexane conversions. Just like any other peroxygenase *AaeUPO* relies on the stoichiometric supply with H₂O₂ as oxidant. It is highly advisable not to provide H₂O₂ all at once at the beginning of the transformation as H₂O₂ is a known inactivator of enzymes, particularly of heme-dependent enzymes [14]. Therefore, a range of in situ H₂O₂ generation systems have been developed in the past decade comprising the in situ reduction of O₂ to H₂O₂ at the expense of a sacrificial reductant [14]. While enabling robust peroxygenase reactions, these methods suffer from the need for a stoichiometric reductant as well as the formation of a stoichiometric by-product.

To alleviate this issue, we conceived a bienzymatic cascade in which an oxidase-catalysed follow-up oxidation of the peroxygenase-reaction produces H₂O₂ as by-product, which in turn can be used as oxidant for the peroxygenase reaction (Scheme 1). Overall, an aerobic oxidation of cyclohexane to cyclohexanone was envisioned.

As alcohol oxidase performing the second oxidation step (and providing the first step with the H₂O₂ needed) we chose a variant of the previously reported alcohol oxidase from *Phanerochaete chrysosporium* (*PcAOx*) [15]. A single point mutation of this enzyme (F101S) had shown some activity towards cyclohexanol [16] but not sufficiently high to be of preparative interest. Therefore, we chose a further engineered variant (*PcAOx*-R61E-Y407F-W560M-N604H, *PcAOx*-EFMH) with increased activity towards cyclohexanol for this study [17]. For the first hydroxylation step we chose the well-known peroxygenase from *Agroclybe aegerita* (*AaeUPO*) [18–21].

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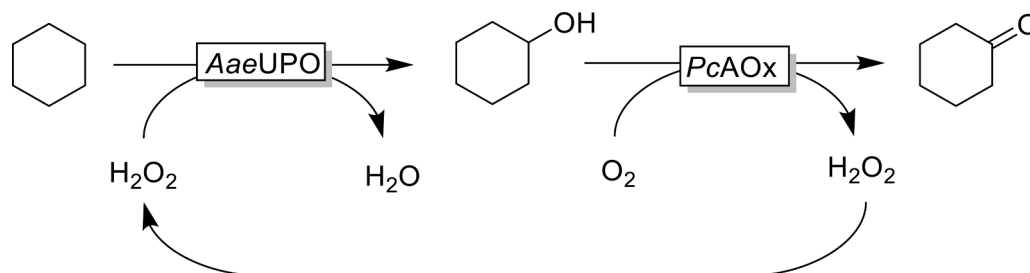
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<https://doi.org/10.1016/j.mcat.2023.113396>

Received 15 April 2023; Received in revised form 17 June 2023; Accepted 15 July 2023

Available online 24 July 2023

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Scheme 1. The envisioned bienzymatic cascade for the aerobic oxidation of cyclohexane to cyclohexanone.

Materials and methods

Chemical reagents and materials

All chemicals were purchased from Sigma-Aldrich, TCI, Oxoid Ltd (UK), Sanland Chemical Co., Ltd or Aladdin in the highest purity available and were used without further purification. *E. coli* BL21 (DE3) was used for the production of the biocatalyst and *E. coli* TOP10 was used for constructing *PcAOx* and its mutants. Water was purified with a Millipore (Bedford, MA) Milli-Q water system.

Construction and expression of the mutant *PcAOx*-EFMH

The mutant was constructed using the wild-type plasmid of AOx as the template. The mutation was completed by site-specific mutation kit and sent to Shanghai Biotech for sequencing. The samples correctly sequenced were carried out for the next experiment. Cells containing target plasmid were cultured to an OD₆₀₀ of 0.7 - 0.8 in 5 mL LB medium containing 50 µg/mL kanamycin at 37 °C and 220 rpm. Then, the cells

were used to inoculate 500 mL TB medium containing 1% glucose, cultured at 37 °C until OD₆₀₀ ≈ 0.8 - 0.9, and induced at 20 °C with 0.5 mM IPTG for 20 h. The cells were collected by centrifugation at 6000 rpm at 4 °C for 30 min and stored at -80 °C for later use.

Enzyme purification for alcohol oxidase mutant *PcAOx*-EFMH

Cells containing recombinant mutant *PcAOx*-R61E-Y407F-W560M-N604H (*PcAOx*-EFMH) were re-suspended in lysis buffer (50 mM potassium phosphate pH 7.5, 400 mM NaCl, 100 mM KCl, 40 mM imidazole, 100 µM FAD) and were ultrasonically broken at 4 °C for 20 min. the ultrasonic pulsed on-time and off-time was 2 s and 3 s, respectively. The cell extract was centrifuged at 4 °C, 11,000 × g for 30 min, and the supernatant was pre-balanced using Ni column with lysis buffer solution. After loading, an imidazole gradient of 40 - 500 mM was used to elute the enzyme. Active fractions were collected and desalted using a desalting column. SDS-PAGE was used to show the enzyme purity. Pure protein was stored at -80 °C for the next experiment.

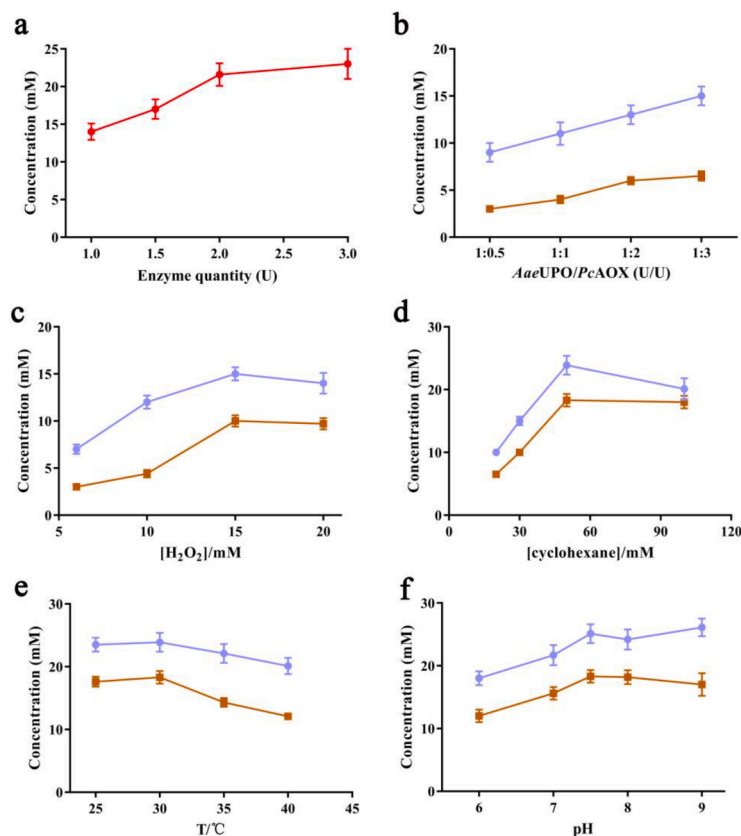


Fig. 1. Influencing factors of UPO cascade *PcAOx* reaction. (●) cyclohexanol, (■) cyclohexanone. (a) Effect of concentration of *AaeUPO* on reaction. (b) Effect of ratio of *AaeUPO* and *PcAOx*-EFMH on the cascade reaction. (c) Effect of concentration of H₂O₂ on the cascade reaction. (d) Effect of concentration of cyclohexane (dissolved in DMSO) on the cascade reaction. (e) Effect of pH on cascade the reaction. (f) Effect of temperature on the cascade reaction. General reaction conditions, unless indicated otherwise: 2 U *AaeUPO*, 4 U *PcAOx*, pH 7.5, 30 °C, 24 h.

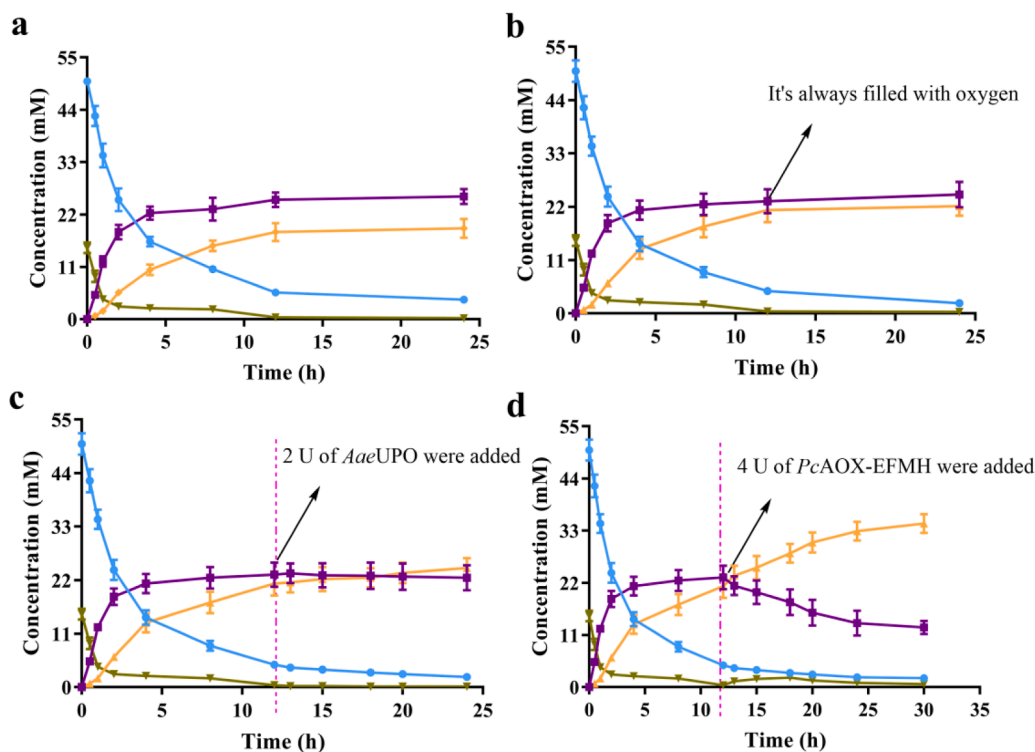


Fig. 2. Time course of the conversion from cyclohexane to cyclohexanone, (●) cyclohexane, (■) cyclohexanol, (▲) cyclohexanone, (▼) H_2O_2 . (a) The lid was not lifted throughout the reaction, (b) Oxygen-filled balloons are used to provide oxygen throughout the reaction. (c) 2 U of *AaeUPO* were added after 12 h. (d) 4 U of *PcAOx-EFMH* were added after 12 h. Reaction conditions: [cyclohexane] = 50 mM (dissolved in DMSO), [H_2O_2] = 15 mM, pH 7.5, 2 U *AaeUPO*, 4 U *PcAOx-EFMH*, 30 °C.

Preparation of UPO

The unspecific peroxygenase from *Agroclybe aegerita* (*AaeUPO*) used in this study was obtained from a previous pilot-scale production of this enzyme [18].

Gas chromatography analysis

The GC-FID analysis was performed on an Agilent 7890B GC system (Agilent Technologies, Palo Alto, CA, USA) outfitted with a 19091J-413 HP-5 column (30 m length \times 0.32 mm I.D. \times 0.25 μm film thickness), and an FID detector. Injection volume: 1 μL ; injection temperature: 280 °C; split ratio: (20:1); detector temperature: 280 °C; initial temperature of the column, 50 °C for 2 min, then increased to 150 °C for 3 min at 8 °C/min; then to 280 °C at 15 °C/min, and maintained for 10 min.

Investigation of factors affecting the cascade reaction

In this cascade reaction, factors affecting the catalytic efficiency of the reaction were investigated, including the amount of UPO (1, 1.5, 2, 3 U), AOx (1, 2, 4, 6 U) and H_2O_2 (5, 10, 15, 20 mM) added, the amount of substrate (20, 30, 50, 100 mM) added, temperature (20, 25, 30, 35 °C) and pH (6, 7, 7.5, 8, 9). To facilitate the solubility of the hydrophobic starting materials in the aqueous reaction mixture 5% (v/v) of DMSO was used. Add all components into a 4 mL reaction flask and fully mix them after 30 s of flushing with O_2 , then tighten the cap of the bottle and then put it into the constant temperature magnetic stirring reaction pot for reaction. The total volume of the reaction system was 1 mL, and the reaction time was 24 h. After the reaction was completed, an equal volume of ethyl acetate was used to extract the products, and the samples was quantified by gas chromatograph. All experiments were conducted in three parallel groups. The blank reaction was performed using

the same volume of buffer instead of enzyme solution.

Results and discussion


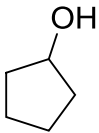
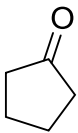

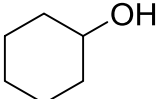
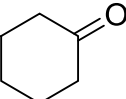
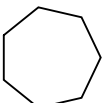
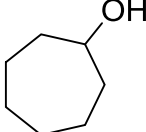
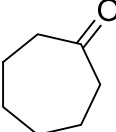
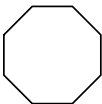
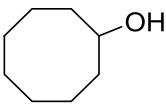
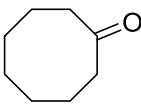
Investigation of influencing factors of cascade reaction

In a first set of experiments we investigated to which extend the peroxygenase catalyst (*AaeUPO*) itself catalysed the individual steps of the cascade (i.e. hydroxylation of cyclohexane to cyclohexanol and the further oxidation to cyclohexanone). We therefore tested the conversion of cyclohexane using different *AaeUPO* concentrations under otherwise identical conditions. As shown in Fig. 1 a, increasing the concentration of *AaeUPO* generally increased the cyclohexanol yield (from 14 mM to 23 mM corresponding to 47 and 77% conversion, respectively). The optimal dosage of *AaeUPO* was 2 U. Interestingly, in none of these experiments significant amounts of cyclohexanone were detectable.

Therefore, we continued and set-up the bienzymatic cascade for the ‘through-oxidation’ of cyclohexane to cyclohexanone by combining *AaeUPO* and *PcAOx-EFMH*. Already under arbitrarily chosen reaction conditions (*AaeUPO*:*PcAOx-EFMH* = 1:2 (i.e. *AaeUPO*: 2 U mL^{-1} , *PcAOx-EFMH*: 4 U mL^{-1})) a significant accumulation of cyclohexanone was observed; also the total amount of product formed (16 mM) exceeded the amount of H_2O_2 added to the reaction system indicating that indeed the proposed in situ H_2O_2 generation (Scheme 1) occurred. Next, we systematically varied the ratio of both enzymes while maintaining the original *AaeUPO* concentration (Fig. 1b). Increasing the concentration of *PcAOx-EFMH* not only increased the concentration of cyclohexanone but also positively influenced the cyclohexanol accumulation. This trend may indicate that the *PcAOx-EFMH*-catalysed oxidation of cyclohexanol and concomitant H_2O_2 formation was overall rate-limiting.

Next, we investigated some other factors influencing the efficiency of the proposed cascade reaction. The initial concentration of H_2O_2 plays

Table 1
Oxygenfunctionalisation of cycloalkanes catalysed by the proposed *AaeUPO*/*PcAOx-EFMH* cascade.

Alkane	Alcohol	Concentration (mM)	Ketone	Concentration (mM)
		17 ± 1		18 ± 2
		13 ± 2		35 ± 2
		9 ± 1		29 ± 2
		13 ± 2		17 ± 2

Reaction conditions: [cyclohexane] = 50 mM (dissolved in DMSO), [H₂O₂] = 15 mM, pH 7.5, 2 U *AaeUPO*, 8 U (4 U/12 h) *PcAOx-EFMH*, 30 °C, 30 h.

an important role in the overall reaction. On the one hand, the higher the kick-starting H₂O₂ concentration, the faster cyclohexanol is formed and can be further oxidised to cyclohexanone. On the other hand, H₂O₂ is detrimental to (heme) enzymes and too high concentrations presumably negatively affect the robustness of the reaction. Under the present reaction conditions, an initial H₂O₂ concentration of 15 mM appeared to represent the optimal value (Fig. 1c). Likewise, increasing the initial starting material concentration (cyclohexane) positively influenced the overall product concentration. E.g. starting with 20 mM cyclohexane (Fig. 1d) resulted in the formation of 10 and 6.5 mM of cyclohexanol and cyclohexanone, respectively (corresponding to 82.5% cyclohexane conversion). Starting with a cyclohexane concentration of 50 mM the final product concentrations were 23.9 and 18.3 mM (corresponding to 84% cyclohexane conversion).

Increasing the reaction temperature above 30 °C negatively influenced the overall reaction (Fig. 1e). Possibly, this observation can be attributed to a combination of decreasing O₂ solubility in the reaction medium and decreased robustness of one or both of the biocatalysts. Similar to temperature also the reaction pH is also an important factor affecting biocatalytic reactions. Interestingly, no clear pH optimum was observed and the overall product formation increased with increasing pH (Fig. 1f). Since the optimal pH value for *AaeUPO* lies in the acidic range [18] this indicates that the *PcAOx-EFMH*-catalysed oxidation of cyclohexanol was overall rate-limiting.

Identification of the overall limitation of the bienzymatic cascade

A typical time course of the UPO-AOx cascade reaction is shown in Fig. 2a. During the initial phase, mostly cyclohexanol formation was observed indicating that the rate of the second oxidation reaction was comparably low. After 4 h of reaction, the concentration of cyclohexanol remained basically unchanged, while the concentration of cyclohexanone was still increasing slowly. We assume that in this phase both reactions (*AaeUPO*-catalysed hydroxylation and *PcAOx-EFMH*-catalysed formation of cyclohexanone) proceeded at the same rates. Finally, after 12 h of reaction, the overall product formation ceased while there was still some starting material present. As this trend was generally observed, we further investigated possible factors limiting the long-term

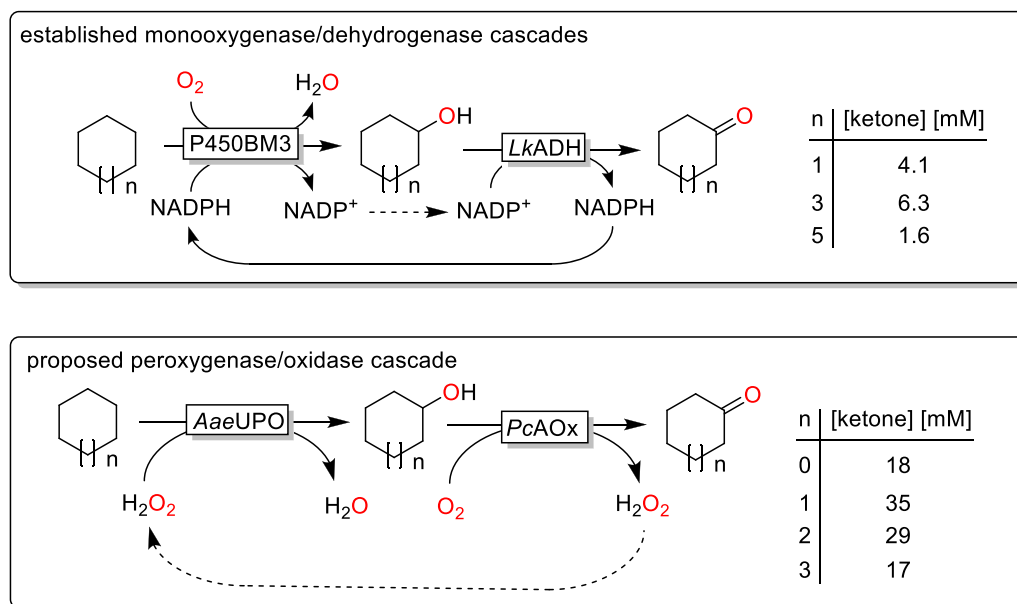
robustness of the reaction. We hypothesised that either O₂-depletion or inactivation of one (or both) of the catalysts may account for the rather poor robustness of the overall reaction. Experiments ensuring additional aeration by means of O₂-filled balloons (Fig. 2b) did not result in a significant prolongation of the reaction. Likewise, further addition of *AaeUPO* after 12 h (Fig. 2c) had only a minor effect. However, addition of further *PcAOx-EFMH* (Fig. 2d) resulted in almost complete conversion of the remaining cyclohexanol. Hence, the robustness of *PcAOx-EFMH* represents the overall limitation of the proposed bienzymatic cyclohexane oxidation scheme. At present we can only speculate about the molecular reason for this apparent instability of *PcAOx-EFMH*. Probably, a poor intrinsic stability of *PcAOx-EFMH* under process conditions seems most likely [18]. However, also a contribution of the H₂O₂ produced cannot be ruled out at this point. Further investigations will clarify this question.

Preliminary scope of cycloalkanes

Encouraged by the above-presented results, we further investigated the substrate scope of the proposed bienzymatic cascade (Table 1). Also some cyclohexane homologs, cyclopentane, cycloheptane and cyclooctane were converted considerably by the proposed cascade. The differences in conversion and ratio of alcohol to ketone most likely originate from different kinetic properties of both enzymes for the starting materials. Further kinetic investigations on both enzymes will enable an in-depth understanding.

Conclusion

Overall, with the present study we demonstrate that a biocatalytic cascade comprising a peroxygenase and an alcohol oxidase may represent a convenient approach for the aerobic oxidation of (cyclo)alkanes to the corresponding ketones such as the conversion of cyclohexane to cyclohexanone. The preliminary results obtained so far (almost 95% conversion of the starting material into cyclohexanol and cyclohexanone) are very promising. Further debottlenecking of limitations in the reaction and use of further engineered biocatalysts will demonstrate the potential of this cascade for the benign synthesis of e.g. polymer



Scheme 2. Comparison of the ketone concentrations reported for a) redox-neutral monooxygenase/dehydrogenase systems and b) the proposed peroxygenase/oxidase cascade.

precursors from simple starting materials. The focus here will lie on understanding the molecular reason for the poor oxidase stability and, based on this, enhancing its robustness under process conditions.

Nevertheless, already at this early stage of development the efficiency of current self-sufficient cycloalkane oxidation cascade compares well with previously reported redox-neutral oxidation cascades comprising NAD(P)H-dependent P450 monooxygenases and NAD(P)H-regenerating dehydrogenases (Scheme 2) [8,9].

Hence, we are convinced that, upon further characterisation and optimisation, the here proposed reaction system bears a significant potential for synthetic application.

Author contribution statement

Bin Wu, Xiangyun Wang and Bo Yang and Yunjian Ma analysed the experimental data. Frank Hollmann assisted in data interpretation and manuscript formulation. Yunjian Ma, Frank Hollmann and Yonghua Wang designed and directed the whole project. All the authors contributed to scientific discussion. The article was written based on contributions from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We sincerely thank Professor Marco W. Fraaije for his helpful comments and guidance on work. This work was supported by the Key Program of Natural Science Foundation of China (grant number 31930084), National Natural Science Foundation of China (32001633). F.H. acknowledges funding by the European Union (ERC, PeroxyZyme, No 101054658). Views and opinions expressed are however those of the

authors only and do not necessarily reflect those of the European Union or the European Research Council. Neither the European Union nor the granting authority can be held responsible for them.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.mcat.2023.113396](https://doi.org/10.1016/j.mcat.2023.113396).

References

- [1] M.T. Musser, Cyclohexanol and cyclohexanone, Ullmann's Encyclopedia of Industrial Chemistry. (2000).
- [2] U. Hanefeld, F. Hollmann, C.E. Paul, Biocatalysis making waves in organic chemistry, *Chem. Soc. Rev.* (2022).
- [3] J. Dong, E. Fernández-Fueyo, F. Hollmann, C.E. Paul, M. Pesic, S. Schmidt, Y. Wang, S. Younes, W. Zhang, Biocatalytic oxidation reactions: a chemist's perspective, *Angewandte Chemie International Edition* 57 (2018) 9238–9261.
- [4] A. Glieder, E.T. Farinas, F.H. Arnold, Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase, *Nat. Biotechnol.* 20 (2002) 1135–1139.
- [5] N. Kawakami, O. Shoji, Y. Watanabe, Use of perfluorocarboxylic acids to trick cytochrome P450BM3 into initiating the hydroxylation of gaseous alkanes, *Angewandte Chemie* 123 (2011) 5427–5430.
- [6] Z. Zhang, Q. Li, F. Wang, R. Li, X. Yu, L. Kang, J. Zhao, A. Li, One-pot biosynthesis of 1, 6-hexanediol from cyclohexane by de novo designed cascade biocatalysis, *Green Chemistry* 22 (2020) 7476–7483.
- [7] L. Bretschneider, I. Heuschkel, M. Wegner, M. Lindmeyer, K. Bühler, R. Karande, B. Bühler, Conversion of Cyclohexane to 6-Hydroxyhexanoic Acid Using Recombinant *Pseudomonas taiwanensis* in a Stirred-Tank Bioreactor, *Frontiers in Catalysis* 1 (2021), 683248.
- [8] S. Staudt, E. Burda, C. Giese, C.A. Müller, J. Marienhagen, U. Schwaneberg, W. Hummel, K. Drauz, H. Gröger, Direktoxidation von Cycloalkanen zu Cycloalkanonen mit Sauerstoff in Wasser, *Angewandte Chemie* 125 (2013) 2415–2419.
- [9] W. Zhang, W.L. Tang, D.I.C. Wang, Z. Li, Concurrent oxidations with tandem biocatalysts in one pot: Green, selective and clean oxidations of methylene groups to ketones, *Chem. Commun.* 47 (2011) 3284–3286.
- [10] D. Holtmann, F. Hollmann, The oxygen dilemma: A severe challenge for the application of monooxygenases? *ChemBioChem* 17 (2016) 1391–1398.
- [11] S. Peter, A. Karich, R. Ullrich, G. Gröbe, K. Scheibner, M. Hofrichter, Enzymatic one-pot conversion of cyclohexane into cyclohexanone: Comparison of four fungal peroxygenases, *J. Mol. Catal. B Enzym.* 103 (2014) 47–51.
- [12] S. Peter, M. Kinne, X. Wang, R. Ullrich, G. Kayser, J.T. Groves, M. Hofrichter, Selective hydroxylation of alkanes by an extracellular fungal peroxygenase, *FEBS J* 278 (2011) 3667–3675.
- [13] E. Churakova, M. Kluge, R. Ullrich, I. Arends, M. Hofrichter, F. Hollmann, Specific photobiocatalytic oxyfunctionalization reactions, *Angewandte Chemie* 123 (2011) 10904–10907.

- [14] B.O. Burek, S. Bormann, F. Hollmann, J.Z. Bloh, D. Holtmann, Hydrogen peroxide driven biocatalysis, *Green Chemistry* 21 (2019) 3232–3249.
- [15] D. Linke, N. Lehnert, M. Nimtz, R.G. Berger, An alcohol oxidase of *Phanerochaete chrysosporium* with a distinct glycerol oxidase activity, *Enzyme Microb. Technol.* 61 (2014) 7–12.
- [16] C. Martin, M. Trajkovic, M.W. Fraaije, Production of hydroxy acids: Selective double oxidation of diols by flavoprotein alcohol oxidase, *Angewandte Chemie International Edition* 59 (2020) 4869–4872.
- [17] B. Wu, S.Y. Wang, Y.J. Ma, S.G. Yuan, F. Hollmann, Y.H. Wang, Structure-Based Redesign of a Methanol Oxidase into an “Aryl Alcohol Oxidase” for Enzymatic Synthesis of Aromatic Flavor Compounds, *J. Agric. Food Chem.* 71 (2023) 6406–6414.
- [18] R. Ullrich, J. Nüske, K. Scheibner, J. Spantzel, M. Hofrichter, Novel haloperoxidase from the agaric basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes, *Appl. Environ. Microbiol.* 70 (2004) 4575–4581.
- [19] F. Tonin, F. Tieves, S. Willot, A. van Troost, R. van Oosten, S. Breestraat, S. van Pelt, M. Alcalde, F. Hollmann, Pilot-scale production of peroxygenase from *Agrocybe aegerita*, *Org. Process. Res. Dev.* 25 (2021) 1414–1418.
- [20] P. Molina-Espeja, S. Ma, D.M. Mate, R. Ludwig, M. Alcalde, Tandem-yeast expression system for engineering and producing unspecific peroxygenase, *Enzyme Microb. Technol.* 73 (2015) 29–33.
- [21] P. Molina-Espeja, E. Garcia-Ruiz, D. Gonzalez-Perez, R. Ullrich, M. Hofrichter, M. Alcalde, Directed evolution of unspecific peroxygenase from *Agrocybe aegerita*, *Appl. Environ. Microbiol.* 80 (2014) 3496–3507.