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ADVANCED SYNTHESIS & CATALYSIS

Constant Enzymatic *in situ* Production of H_2O_2 for an Unspecific Peroxygenase by an L-Amino Acid Oxidase

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Abstract: Unspecific Peroxygenases have been the subject of a lot of research in the recent past as they offer opportunities for green hydroxylation reactions using hydrogen peroxide (H_2O_2) as donor. A striking problem of UPOs is their low stability against their own co-substrate H_2O_2. In order to prevent the deactivation, we developed an enzyme cascade with an L-amino acid oxidase (LAAO) to produce hydrogen peroxide *in situ* at a constant low concentration, aiming for much higher lifespan and therefore longer working time of the enzyme. This way, we are able to convert a variety of substrates with conversion rates up to 95%.

Keywords: Enzyme cascade; Enzyme stability; Hydrogen peroxide; Oxyfunctionalisation; Unspecific Peroxygenases (UPO)

Introduction

Oxyfunctionalised compounds are of great interest as unique intermediates for the pharmaceutical industry.[1–4] In order to stereoselectively synthesize a wide range of these valuable substances, biocatalysis provides variety of unique pathways to obtain the desired products. The usage of enzymes as catalysts opens up a range of new possibilities for the synthesis of otherwise difficult-to-access chemical compounds in high purity under mild conditions.[5] As the use of environmentally harmful, toxic and expensive chemicals can usually be dispensed with, biocatalysis represents an attractive approach to green process design and economic workflows. Moreover, enzymes can easily be customised through the systematic genetic modification and generally easily produced via certain organisms such as *E. coli* or yeast.[6–8]

Unspecific Peroxygenases (UPOs, EC 1.11.2.1) are a representative of this group of biocatalysts.[3,9,10] They were first described in 2004 as a new form of haloperoxidases.[11] As originally fungal enzymes, UPOs can be obtained by expression in yeast and catalyse selective oxyfunctionalisation reactions, such as the enzymatic hydroxylation of sp^3-hydrogen-carbon bonds.[12–14] Instead of expensive co-factors, they only require hydrogen peroxide (H_2O_2) as a co-substrate, which is considered a mild and environmentally friendly oxidising agent. Similar to P450 monooxygenases that have been known for quite some time, UPOs belong to the haem-thiolate proteins. These enzymes coordinate the hydrogen peroxide to the Fe^{3+} in their active site, forming the actual active Fe^{4+} species, This radical Fe^{4+} species leads to homolytic cleavage of a C–H bond, which in turn produces a radical that accepts the oxygen from the active centre.[15,16] In this

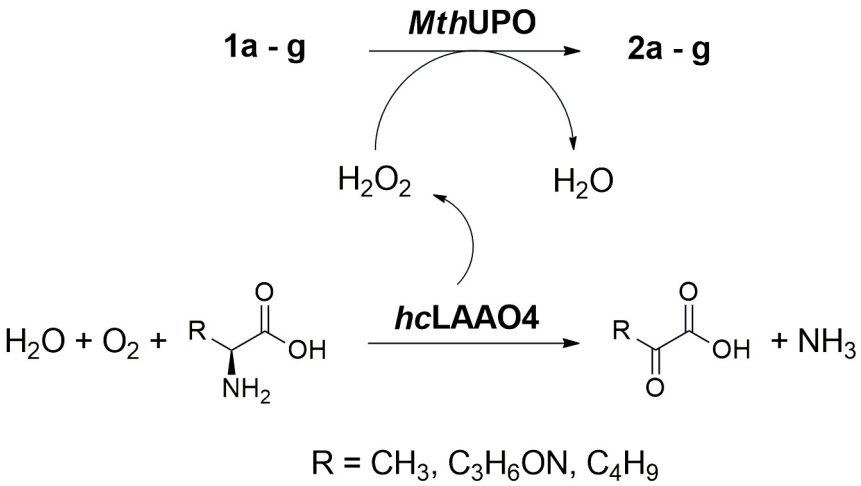


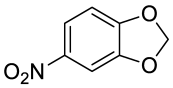
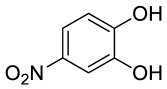
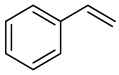
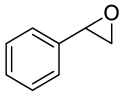
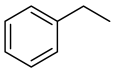
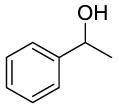
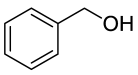
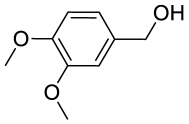
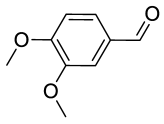
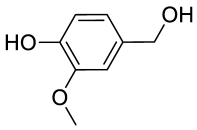
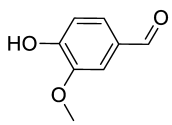
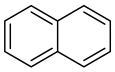
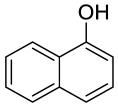
Figure 1. *Mth*UPO-catalysed oxyfunctionalisation with enzymatic *in situ* H_2O_2 production.

way, UPOs are able to carry out oxyfunctionalisations, e.g. the epoxidation of styrene and the synthesis of amino alcohols from amines.[12,15,17,18] Nonetheless, UPOs unfortunately tend to get inactivated in the presence of their own co-substrate, H_2O_2, mainly as a result of a Haber-Weiss reaction.[19,20] Therefore, the concentration of H_2O_2 needs to be well controlled during UPO-catalysed reactions. The concentration must be kept low. A possible solution to that problem is the *in situ* production of H_2O_2. Multiple approaches were described using the anthraquinone process, photo- and electrochemical or techniques that use nuclear radiation.[21–27] A cascade with a second enzyme could be an easy-to-control and elegant alternative.

Therefore, this study focuses on the *in situ* generation of H_2O_2 through the L-amino acid oxidase from *Hebeloma cylindrosporum* (*hc*LAAO4, EC 1.4.3.2), which converts L-amino acids to α-keto acids and ammonia, generating H_2O_2 as a side-product (Figure 1).[28–30] Thus, constant and *in situ* produced H_2O_2 could be applied at low concentrations by adjusting the addition of the L-amino acids.[31] As a model enzyme, the UPO from *Myceliophthora thermophila* (*Mth*UPO) was chosen because it can be used for a wide variety of substrates with activated and inactivated sp^3-hydrogen-carbon bonds as well as different alcohols (Table 1).[8,12,32,33]

Figure 1 demonstrates a concept for a *Mth*UPO-catalysed oxyfunctionalisation with enzymatic *in situ* H_2O_2 production. In the presented enzyme cascade, *hc*LAAO4 oxidises L-α-amino acids to α-keto acids, thereby constantly producing H_2O_2 as a by-product. The latter is used as co-substrate by the applied *Mth*UPO to hydroxylate different substrates to their corresponding products (Table 1).

Table 1. Investigated substrates and their respective main products.

	Substrate **1**	Main product **2**
a		
b		
c		
d		
e		
f		
g		

Results and Discussion

H_2O_2-stability of *Mth*UPO

The stability of *Mth*UPO towards its own co-substrate, H_2O_2, especially at higher concentrations, is relatively low and thus a careful control is necessary to maintain an active enzyme, particularly at preparative scales

with higher substrate and thus higher H_2O_2 concentrations (Figure 2).

To discover which concentrations of H_2O_2 are detrimental to *Mth*UPO, the enzyme was incubated with different concentrations of H_2O_2 prior the standard reaction. The activity of *Mth*UPO was maintained up to a concentration of only 1 mM H_2O_2 with 443 and 448 mU/ml for 0.5 and 1.0 mM H_2O_2, respectively. This is in range of the observed K_M-value for H_2O_2 in the reaction with NBD.[12] Higher concentrations led to the deactivation of the enzyme with 43 mU/ml at 2.0 mM and a complete loss of activity at 2.5 mM. This might be due to a Haber-Weiss reaction, which effectively disintegrates the active site of enzyme in an irreversible fashion. Interestingly, increasing H_2O_2 concentrations resulted in a faster deactivation mechanism, which proves the importance of a well dosed addition of H_2O_2 at low concentrations and a very small window of operation (Figure 2). Only the lowest concentrations of hydrogen peroxide allowed the activity of the *Mth*UPO to be maintained.

Implementation of *hc*LAAO4 for H_2O_2 Generation

The proposed approach for a constant, low concentration of H_2O_2 for the *Mth*UPO-catalysed reaction utilises a second enzyme in a biocatalytic cascade. The L-amino acid oxidase from *Hebeloma cylindrosporum* (*hc*LAAO4) was chosen, which converts a wide range of L-amino acids into α-keto acids, simultaneously producing ammonia and H_2O_2 in the process.[28] This enzyme is highly stable towards H_2O_2, provides high activity after activation and can easily be produced at a larger scale compared to other LAAOs.[29,30]

Since a too rapid production of H_2O_2 would also lead to the deactivation of the *Mth*UPO, various concentrations of the highly active *hc*LAAO4 were tested. It was observed that even slightly too high concentrations of LAAO led to an overproduction of H_2O_2, resulting in the termination of the UPO reaction. As the *hc*LAAO4 exhibits a significantly higher activity than the *Mth*UPO, the amounts of both enzymes were coordinated by pre-diluting the LAAO, so that the activities of the enzymes were similar to each other. To avoid too high concentrations of the L-amino acid as a co-substrate, which would result in overproduction of H_2O_2, different concentration ranges and L-amino acids were analysed. Importantly, equimolar amounts of the respective substrate and L-amino acid were found to be optimal, since H_2O_2 is quickly consumed by the applied *Mth*UPO that way. After evaluating the *hc*LAAO4-based *in situ* H_2O_2 generation concept, the overall conditions were transferred to more synthetic applications. Using the model compound 3,4-methylenedioxynitrobenzene (**1a**), the total lifespan of the *Mth*UPO was investigated, which increased significantly using the cascade option (Figure 3). Thereby, the conversion rate could be increased from only 13% to 58% for the exemplary conversion of **1a** to **2a**. The lifespan of *Mth*UPO could be extended from a few minutes using an external H_2O_2-source into the range of hours via the proposed biocatalytic reaction.

Furthermore, it was found that the broad substrate spectrum of *hc*LAAO4 allows a wide applicability for

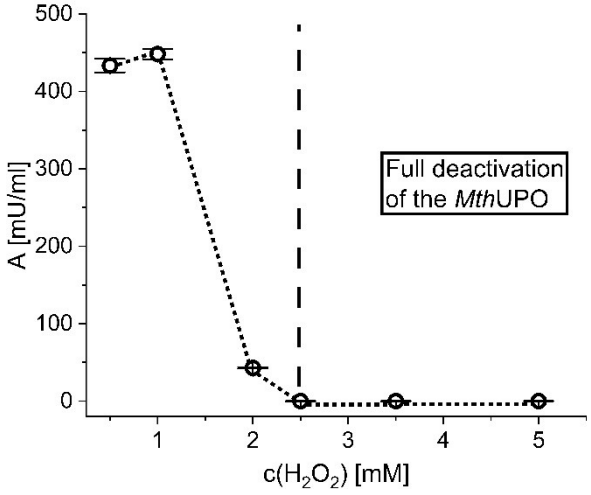


Figure 2. Effect of H_2O_2 on the activity of *Mth*UPO (starting activity of 1.44 U/ml), ten minutes of incubation at 30 °C after adding different amounts of H_2O_2 to the reaction media; conditions of the NBD assay: 0.5 mM **1a**, 0.1 M HEPES (pH 7.0), different concentrations of H_2O_2, volume adjusted with ultrapure water to a total volume of 200 µl; measurement at 425 nm in triplicates.

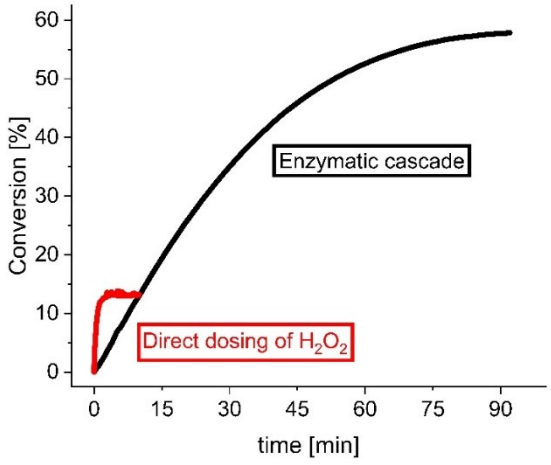


Figure 3. Comparison of the conversion of **1a** to **2a**; with direct dosing of H_2O_2 (red) and the cascade reaction with *hc*LAAO4 (black); 0.5 mM **1a**, 0.1 M HEPES (pH 7.0), 10 µl *Mth*UPO (1.44 U/ml), for direct dosing 1 mM H_2O_2, 1.05 µg/ml *hc*LAAO4 for the cascade, the volume in each case was adjusted with ultrapure water to a total volume of 200 µl, measurement at 425 nm in triplicates.

a range of L-amino acids with different affinities and thus activities of the LAAO.[28–30] L-alanine, L-glutamine and L-norleucine were investigated as substrates for *hc*LAAO4 in the enzyme cascade. All applied amino acids resulted in similar *Mth*UPO-activities of 318, 376 and 361 mU/ml, respectively (Figure 4). This demonstrates the ease of use for a range of applications, even with specific amino acid requirements, because the affinity of *hc*LAAO4 was proved to be very high for almost every L-amino acid before.

As mentioned before, increasing the concentration of the amino acid would lead to a rapid overproduction of H_2O_2, thereby quickly deactivating the applied *Mth*UPO. This could be avoided by a careful design control of the amino acids in relation to the *Mth*UPO substrate (Figure 2 in comparison). By coordinating the amounts of both enzymes with each other as well as adding equimolar amounts of L-amino acids to the reaction solution, the observed overall activity of the cascade for the conversion of **1 a** turned out to be smaller than the originally determined 400 mU/ml, but the lifespan of the *Mth*UPO can be increased significantly, which leads to a more complete turnover. Nonetheless, the LAAO reaction is the speed-determining step of the cascade, as it produces the co-substrate required by the *Mth*UPO. By carefully tuning the amounts of both enzymes and the ratio of both substrates, a balance can be achieved between stability and reaction speed to ensure as complete a conversion as possible.

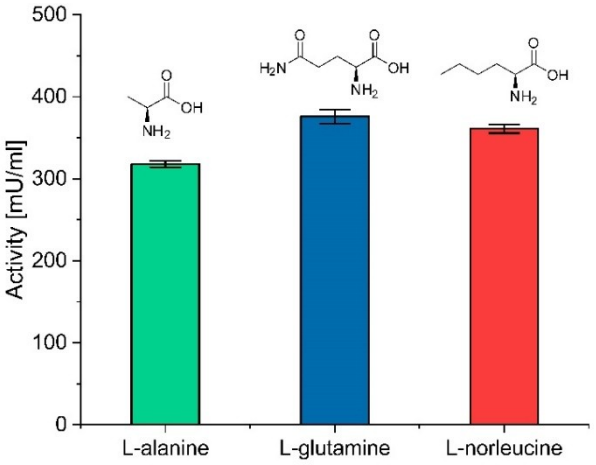


Figure 4. *Mth*UPO activities with different L-amino acids applied in the enzyme cascade. 10 mM of either L-alanine, L-glutamine and L-norleucine as substrate for the *hc*LAAO4 to produce H_2O_2, 0.1 M HEPES (pH 7), 1.05 µg/ml *hc*LAAO4, 10 µl *Mth*UPO (1.44 U/ml) and 0.5 mM **1 a**, the volume in each case was adjusted with ultrapure water to a total volume of 200 µl, measurement at 425 nm in triplicates.

Application of the *Mth*UPO-*hc*LAAO4-cascade

To translate the findings observed for the conversion of **1 a** by the established enzyme cascade, different substrates were applied to the reaction (Table 2). All substrates were accepted by the enzyme without deactivating it and high conversions (> 80%) were mostly observed. It could thereby be demonstrated that *hc*LAAO4 can be added to the reaction independent of the UPO substrate and is only necessary for H_2O_2 supply.

To highlight the synthetic potential of the cascade system using *Mth*UPO and *hc*LAAO4, a series of substrates was chosen. This included the originally applied model compound **1 a**, which is commonly used for the photometric determination of peroxygenase activity, and was extended to a wide range of other starting materials. The *Mth*UPO firstly oxyfunctionalises the methylene carbon of **1 a**, followed by a decarboxylation, which represents an advantage of this reaction to **2 a**, leading to an additional shift to the product side.

Styrene (**1 b**) showed a good conversion that was even exceeded by the conversion rates of **1 c** and **1 d**. Both were converted to nearly 90%; it can be assumed that the different activation of the benzylic position is the reason for this behaviour. An overoxidation of **2 c** and **2 d** due to an excess of H_2O_2 was not observed, as expected from the *Mth*UPO.[12] In case of **2 b**, a variety

Table 2. Conversion of different substrates tested for *Mth*UPO in this work.

	Substrate **1**	Product **2**	Conversion [%]
a	3,4-Methylene-dioxynitro-benzene	4-Nitrobenzene-1,2-diol	81.4
b	Styrene	Styrene oxide	82.3
c	Ethylbenzene	1-Phenylethanol	87.9
d	Toluene	Benzylic alcohol	88.9
e	Veratrylic alcohol	Veratrum aldehyde	48.8
f	Vanillyl alcohol	Vanillin	23.7
g	Naphthalene	1-Naphthol,	94.6

Reaction conditions: 100 µl *Mth*UPO (1.44 U/ml) solution, 100 µl *hc*LAAO4 solution (21 µg/ml), 0.1 M HEPES buffer (pH 7.0), 20 mM L-alanine solution (in 0.1 M HEPES buffer pH 7.0), 20 mM of either substrate, adjusted to a total volume of 1 ml with ultrapure water. Measurements took place over a time of 4 hours in triplicates.

of side reactions were observed, leading to different ring-opening products and even polymers (see Supporting Information). The reason for this behaviour lies in the ring tension of the three-membered epoxide ring, which can easily be opened due to reactions with a wide variety of reaction partners. Because the reaction medium contained definite amounts of different acids and rising amounts of α-keto acids due to the *hc*LAAO4 reaction, the ring opening reaction can easily occur. The same is true for water being a nucleophilic compound itself, forming ring opening products with **2 b** over time, too.

Moreover, substances with high boiling points were observed in the reaction with **1 b** using gas chromatography (see Figure S12). As commonly known, both styrene and styrene oxide are considered monomers for the building of polymers. Because the reaction mechanism of the oxyfunctionalisation by *Mth*UPO is one with radical species involved[12,15], it seems convincing that small portions of **1 b** and **2 b** can be radicalised in order to start a polymerisation reaction, leading to consumption of undefined quantities of both compounds. A cloudy white precipitation could be observed in the reaction medium on several occasions, before and after the extraction step, which appeared in larger quantities the longer the reaction lasted.

For substrates **1 e** and **2 f** the conversion seemed to be less effective with conversion rates of 48.8 and 23.7%, respectively. A possible explanation for this observation seems to lie in the different structure of the substrates. It seems that the *Mth*UPO simply displays lower activities for both those substrates, resulting in lower conversion rates.[12] Nevertheless, the enzymatic cascade allowed the conversions of these two relatively low-affinity substrates, producing *in situ* H_2O_2 for the oxyfunctionalisation of **1 e** and **1 f**.

In addition, **1 g** showed the highest conversion (94.6%) of all tested substrates. However, despite the use of the cascade, overoxidation of **2 g** was observed, leading to the formation of 1,4-naphthoquinone, which made up a not to be underestimated proportion of the obtained products (compare Figure S3). This could not be prevented, as the *Mth*UPO has a high affinity for these substances. Both resulting compounds could be detected, using NMR analysis. The same applies to some species formed by the reaction of **2 g** and its by-product with different acids contained in the reaction solution. Using gas chromatography, numerous compounds with high boiling points were observed, which unfortunately could not be isolated for further investigations, but it is very likely that these compounds are esters or keto esters of **2 g**. For this reason, the conversion of **1 g** seems to be – although promising at first glance – mostly driven by the follow-up reactions of the desired product **2 g**. Even though the influence of the pH value could be considered a factor in these observations, due to the high buffer concentration, which exceeded see acid concentrations by far, pH values remained constant and these interferences could be excluded. In terms of the overoxidation of **1 g**, the *Mth*UPO seems to possess a high affinity for this specific substrate and its product, especially since 2 g also has an activated benzylic position, which simplifies the further oxyfunctionalisation of the aromatic compound.[12] This behaviour was also observed in experiments with direct dosing of H_2O_2.

The capability for the decarboxylation of α-keto acids in the presence of H_2O_2 is a well-known phenomenon. The decay reaction only takes place after a certain accumulation time and only above a critical concentration of H_2O_2. This behaviour was tested on both a small assay-level and a larger technical scale (see Figure S13). In those cases, a strongly exothermic evolution of carbon dioxide was observed, resulting in carboxylic acids with a chain length one carbon shorter than the respective α-keto acid. These keto acids are formed from L-amino acids as a product of the *hc*LAAO4 reaction and are considered highly reactive. Therefore, it is possible to see reactions of not only the acids used, but also the corresponding α-keto acids and their decay products.

However, in case of relatively fast consumption of the generated hydrogen peroxide through the *Mth*UPO, the decarboxylation was not observed to be a significant issue. Only in some instances (products **2 b**, **2 c** and **2 g**), several by-products were observed, which were attributed to the presence of the of the L-amino acid used and the respective α-keto acid. Fortunately, the *Mth*UPO was shown to quite selectively oxyfunctionalise the benzylic position of respective substrates or respectively inactivated positions of the chosen substrates. For this reason, **1 e** and **1 f** could naturally not be converted at comparably high rates as the substrates which contain benzylic carbons. Of course, the reaction will end as soon as all of the L-amino acid is consumed by the *hc*LAAO4 and therefore there would be no longer any production of hydrogen peroxide.

A decisive advantage of the cascade is the possibility of using higher substrate concentrations. As the hydrogen peroxide is produced *in situ* instead of being supplied directly, the enzyme is active for a much longer time and generates higher overall conversions compared to direct dosing.

Summary and Conclusion

Unspecific Peroxygenases (UPOs) offer a great synthetic approach to selective oxidation reactions, but simultaneously suffer from the general issue that their own co-substrate hydrogen peroxide can inactivate the enzyme. Therefore, low concentrations of H_2O_2 should be maintained and constantly supplied to avoid rapid inactivation of the enzyme. This study demonstrates

the design of an enzymatic cascade, which significantly extends the lifespan from minutes to hours of the applied *Mth*UPO. The L-amino acid oxidase from *Hebeloma cylindrosporum* was chosen to supply H_2O_2 *in situ* at low concentrations to facilitate the oxidation reaction of the investigated *Mth*UPO with simultaneous prevention of inactivation. This system could be transferred to reactions with multiple substrates, including otherwise often hard to convert non-activated compounds, with a significant improvement of the half life time of the applied *Mth*UPO. The shown cascade reaction is highly practical and an easy-to-use alternative to other already established techniques for *in situ* H_2O_2 generation, particularly at the preparative scale of up to 20 mM. Using the cascade system helps to maintain the stability of the *Mth*UPO and opens up various avenues for future applications.

Experimental Section

General information. All chemicals and solvents used for synthesis, cell expression, processing and analytical purposes were purchased from commercial distributors of analytical grade – Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Thermo Fisher Scientific (Waltham, MA, USA). Additionally, SC Drop-Out for the expression media for yeast production were purchased from Formedium LTD (Kings Lynn, England).

Transformation, expression and concentration of *Mth*UPO. Chemical competent cells of *S. cerevisiae* (INVSc1 strain) were used for the transformation using polyethylene glycol/lithium acetate. Therefore, chemically competent INVSc1 cells were prepared in transformation buffer (15% (v/v) glycerol; 100 mM lithium acetate; 500 µM EDTA; 5 mM Tris-HCl pH 7.4). For each transformation, 100 ng of prepared plasmid pAGT572 Nemo2.0 *Mth*UPO[12] were mixed with 10 µl of pure single stranded salmon sperm DNA (Merck, D7656-1 ML) and added to 200 µl of INVSc1 cells on ice. Afterwards, 600 µl of transformation buffer (40% (v/v) polyethylene glycol 4000; 100 mM lithium acetate; 1 mM EDTA; 10 mM Tris-HCl pH 7.4) were added and the cells were incubated under shaking (850 rpm, 30 °C) for 30 minutes. Before the heat shock at 42 °C for 15 minutes, 70 µl DMSO were added to the mixture. The cells were pelleted by a short centrifugation and the supernatant was discarded. The cell pellet was resuspended in 350 µl of sterile ultrapure water. 100 µl were plated on Synthetic Complement (SC) Drop Out plates, containing 2% (w/v) glucose as carbon source and lacking Uracil as selection marker. The plates were incubated for at least 48 h at 30 °C until white colonies appeared. Single colonies were used to inoculate 100 ml SC Drop Out medium, containing raffinose as carbon source, in a sterile erlenmeyer flask. The addition of 25 µl chloramphenicol (50 mg/ml in ethanol) ensured a clean growth of yeast cells without unwanted foreign organisms. After incubating for three days (130 rpm, 30 °C), the optical density of the solution was measured at 600 nm. The main cultures contained 333 ml 1,5x YP (3% (w/v) peptone, 1.5% (w/v) yeast extract), 50 ml D-galactose (20% (w/v)), 16.5 ml pure ethanol, 36 ml KPi buffer (1 M, pH 6.0), 16 ml $MgSO_4$ (100 mM), 48 ml sterile ultrapure water and 0.25 ml chloramphenicol stock solution (50 mg/ml). After adding suitable amounts of the pre-culture, so that the solution reached an optical density of 0.3 at 600 nm, the main cultures were incubated for three days at 120 rpm and 25 °C. Afterwards, the solutions were centrifugated (3500 rpm, 4 °C, 30 min) and the cells discarded. In order to increase the concentration, the solution went through ultrafiltration using a Vivaflow 200 filtration module (30 kDa, PES, Sartorius). Finally, the dark-brown supernatant was sterile filtrated and stored at 4 °C.

Preparation of *hc*LAAO4. The L-amino acid oxidase from *Hebeloma cylindrosporum* was expressed in *E. coli* and purified according to literature by the working group of Gabriele Fischer von Mollard.[29,30]

Enzymatic reactions. In order to oxyfunctionalise a given substrate, the Unspecific Peroxygenase needed to be provided with small amounts of hydrogen peroxide. Therefore, equal volumes of L-amino acid oxidase solution were added to any reaction solution. Because of its extensively higher activity the *hc*LAAO4 had to be pre-diluted in a factor of one thousand, so that in each assay and reactor its final concentration was reduced to 1.05 µg/ml. *Mth*UPO and *hc*LAAO4 were dosed in a way that their respective volume made out one twentieth of the total volume. The photometric observed assays were fulfilled in a 200 µl scale, while most reactions were performed in a volume of 1 to 10 ml, with HEPES buffer (100 mM, pH 7.0) and respective L-alanine, L-glutamine or L-norleucine as amino acid substrate for the *hc*LAAO4. The catalytic capability of the *Mth*UPO was tested with 5-nitro-1,3-benzodioxole, veratrylic alcohol, styrene, toluene, naphthalene, vanillyl alcohol and ethyl benzene. First test reactions of each substrate were performed using 0.5 mM of each substance, for testing the capability of the *Mth*UPO to convert it. Afterwards, higher substrate concentrations were validated in regard of the stability and activity of the peroxygenase. Samples were taken periodically, deactivated using hydrochloric acid and extracted with dichloromethane to be analysed via gas chromatography. To examine the respective conditions for the combination of *Mth*UPO and *hc*LAAO4, several parameters like enzyme concentration and pre-dilution, different amino acids, buffers and co-solvents for substrates, and various substrates in different concentrations had been validated. Assays and reactions were run in durations from a few minutes up to several hours and even days to observe the behaviour of enzyme, substrate and product.

Photometer (TECAN plate reader). All photometric measurements were performed on a Spark Multimode Microplate Reader (Tecan Trading AG, Switzerland). For absorbance measurements a xenon flash lamp was used, with a spectral range from 200 to 1000 nm, an optical density range from 0 to 4 OD, a scan speed $\leq$ 5 sec and a wavelength accuracy of $<$ 0.3 nm. Microplates (96 well, PS, F-Bottom, clear) of the company Greiner Bio-One were used for every experiment.

NBD Assay. 5-nitro-1,3-benzodioxole (NBD) can be used as a substrate for activity measurements for *Mth*UPO. It is converted to 4-Nitrocatechol, which is measured at a wavelength of 425 nm. The reactions were performed in above mentioned 96 well plates. A total volume of 200 µl per well contained 0.5 mM NBD (stock solution of 10 mM, solved in acetonitrile), 10 µl of concentrated peroxygenase containing supernatant

(1.44 U/ml equals 0.072 U/ml in the final solution), 10 µl *hc*LAAO4-solution or H_2O_2 solution, 0.5 mM amino acid (stock solution of 10 mM, solved in ultrapure water; L-alanine, L-glutamine or L-norleucine for instance), 100 mM HEPES buffer pH 7.0 and were to be filled up with ultrapure water. Measurements were performed over durations from a few minutes up to eight hours, paying most attention to the linear segment of each run (usually 2 min). The slope of each measurement was used to determine the activity of the respective enzyme under the given circumstances. All reactions were performed as triplicates.

H_2O_2 Assays. The concentration of hydrogen peroxide can be measured with different assays, using a photometer. An assay with ammonium molybdate and potassium hydrogen phthalate can be chosen for the determination of solutions with moderate concentrations of H_2O_2, measuring at a wavelength of 351 nm. Therefore, 60 µl colour solution (0.4 M KI, 0.05 M NaOH, 10^{-4} M ammonium molybdate) and 60 µl potassium hydrogen phthalate solution (0.5 M) were mixed before adding 80 µl sample solution containing hydrogen peroxide (10 – 100 µM). The system was calibrated using precise H_2O_2 standard solutions.

Similarly, a POX/o-Dianisidin assay was used to measure the activity of L-amino acid oxidase (LAAO) as well as the H_2O_2 concentration of certain solutions. In a total volume of 200 µl, 10 mM L-alanine (stock solution of 100 mM, solved in ultrapure water), 1.25 µl (0,2 mg/ml) o-Dianisidin (stock solution of 32 mg/ml, solved in DMSO), 2.5 µl peroxidase solution (POX, 400 U/ml), and 10 µl sample were mixed in TEA buffer (50 mM, pH 7.0) and measured at a wavelength of 436 nm for twenty minutes at 30 °C.

GC-FID. For product quantification and purity verification, calibration curves were created, using a Nexis GC – 2030 from Shimadzu Europa GmbH (Duisburg, Germany). The device was equipped with an AOC-20i Plus Auto Injector and a SH-5 column (P/N 221–75701-30, length 30 m, ID 0.25, DF 0.25, temperature range −60 to 330/350 °C). The stationary phase of the column is composed of 5% diphenyl polysiloxane and 95% dimethyl polysiloxane. Hydrogen was the main carrier gas with a flow rate of 36.0 ml/min. Nitrogen was used as make-up gas (24.0 ml/min), and compressed air (200.0 ml/min) for the flame ionisation detector (FID). Samples were converted in aqueous solution, deactivated with hydrochloric acid and extracted with dichloromethane or CPME, respectively. The temperature program was driven from 100 to 200 °C with a heating rate of 10 °C/min, followed by a holding step over two minutes, and in a second heating step up to 250 °C with a heating rate of 25 °C/min over a total time frame of sixteen minutes.

HPLC. As another method of quantification and verification of purity, High Performance Liquid chromatography measurements were performed using a device from Shimadzu Europa GmbH (Duisburg, Germany). The HPLC was equipped with a SCL-40 system controller, a SIL-40 C auto sampler and a LC-40D solvent delivery module. A CTO-40S column oven was used to temper the column to 40 °C throughout the measurement. To provide information about the contained samples, a SPD–M40 photo diode array detector measured in a wavelength area from 190 to 450 nm. The program was isocratic; over a range of 25 minutes and with a flow rate of 0.08 ml/min, the mobile phase consisting of 65% 10 mM phosphate buffer pH 4.0 and 35% MeOH/MeCN (1:1) was driven through a C18 LC column (Kinetex, 150 x 3 mm, 2.6 µm, 100 Å). Per sample a volume of 10 µl was injected. Each sample taken from a reaction solution containing any type and amount of enzyme was processed in the same way. To 1 ml of the solution taken from the reaction vessel 50 µl of concentrated hydrochloric acid was added, in order to stop any enzymatic process. Afterwards, 250 µl of this solution were mixed with 1 ml of MeOH/MeCN (1:1) and centrifuged for three minutes. 525 µl of the supernatant were given to 975 µl of 10 mM phosphate buffer pH 4.0.

Nuclear magnetic resonance (NMR). The NMR spectra were recorded on an AV III Bruker-BioSpin (^{1}H: 400.13 MHz; ^{13}C: 100.62 MHz) or a Avance Neo Bruker BioSpin (^{1}H: 600.13 MHz; ^{13}C: 150.9 MHz). ^{1}H and ^{13}C shifts were referenced to internal solvent resonances and reported in parts per million relative to TMS. The solutions were prepared respectively in CD_3OH and $CDCl_3$.

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