



# Natural Deep Eutectic Solvents as Performance Additives for Peroxygenase Catalysis

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Natural deep eutectic solvents (NADES) are proposed as alternative solvents for peroxygenase-catalysed oxyfunctionalization reactions. Choline chloride-based NADES are of particular interest as they can serve as solvent, enzyme-stabiliser and sacrificial electron donor for the *in situ* H<sub>2</sub>O<sub>2</sub> generation. This report provides the first proof-of-concept and basic characterisation of this new reaction system. Highly promising turnover numbers for the biocatalysts of up to 200,000 have been achieved.

Water is the solvent of biocatalysis; the vast majority of biocatalytic reactions reported today take place in aqueous reaction media. Apparently, this is a direct consequence of the

importance of water for life and the fact that most enzymes (the catalysts of life) are readily soluble in aqueous media.

In many aspects, however, water is not an ideal solvent for chemical transformations utilising enzymes. Most reagents of interest, for example, are rather hydrophobic and therefore only sparingly soluble in aqueous reaction media. Also, it should be noted that the stability of enzymes in non-aqueous media can significantly be higher than in aqueous media.<sup>[1]</sup> Therefore, the quest for alternative solvents enabling higher reagent loadings and improved biocatalyst performance, is ongoing and gaining momentum.<sup>[2]</sup> In 2003 Abbot and co-workers first reported a novel class of eutectic mixtures, termed Deep Eutectic Solvents (DES),<sup>[3]</sup> which ever since have found wide interest as simpler and environmentally more acceptable alternatives to ionic liquids.<sup>[4]</sup> Shortly afterwards some of us discovered that DES occur naturally (termed Natural Deep Eutectic Solvents, NADES).<sup>[5]</sup> Not very astonishingly, (NA)DES have also been evaluated as solvents for biocatalytic reactions.<sup>[2d]</sup> The majority of studies has been focusing on hydrolase-catalysed (trans) esterification reactions while the application of NADES on further enzyme classes is still in its infancy.<sup>[2d]</sup>

We therefore set out to explore the potential of NADES as 'performance solvents' for peroxygenase-catalysed oxyfunctionalisation reactions. Peroxygenases are a new class of heme-thiolate dependent enzymes catalysing P450-monooxygenase-like oxyfunctionalisation reactions<sup>[6]</sup> but, unlike P450 monooxygenases, do not rely on complicated electron transport chains. Instead, peroxygenases utilise H<sub>2</sub>O<sub>2</sub> as stoichiometric oxidant. However, if applied in surplus H<sub>2</sub>O<sub>2</sub> leads to oxidative inactivation of the biocatalysts. Therefore, a range of *in situ* H<sub>2</sub>O<sub>2</sub> generation approaches have been developed to balance the H<sub>2</sub>O<sub>2</sub> concentration to levels where the desired peroxygenase reaction rate is maximised while the undesired oxidative inactivation rate is minimised.<sup>[7]</sup>

Here, we propose an *in situ* H<sub>2</sub>O<sub>2</sub> generation system based on Choline oxidase (ChOx)-catalysed conversion of choline into betaine and the concomitant generation of two equivalents of H<sub>2</sub>O<sub>2</sub> to drive peroxygenase-catalysed oxyfunctionalisation (Scheme 1). This way, we envision utilising ChCl-based NADES both as solvent and as sacrificial electron donor at the same time.<sup>[8]</sup>

As the biocatalysts, we chose the choline oxidase from *Arthrobacter nicotianae* (AnChOx),<sup>[9]</sup> and the recombinant, evolved peroxygenase from *Agroclybe aegerita* (rAaeUPO).<sup>[10]</sup> Both enzymes were produced by heterologous expression in *Escherichia coli* or *Pichia pastoris*, respectively, following estab-

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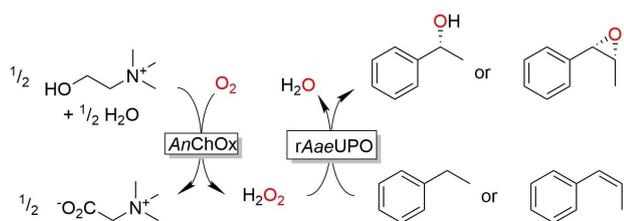
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**Scheme 1.** Envisioned bienzymatic cascade for the selective oxyfunctionalisation of ethyl benzene or *cis*- $\beta$ -methylstyrene comprising the evolved peroxygenase from *Agroclybe aegerita* (*rAaeUPO*) and choline oxidase from *Arthrobacter nicotianae* (*AnChOx*) to provide *rAaeUPO* with  $\text{H}_2\text{O}_2$ .

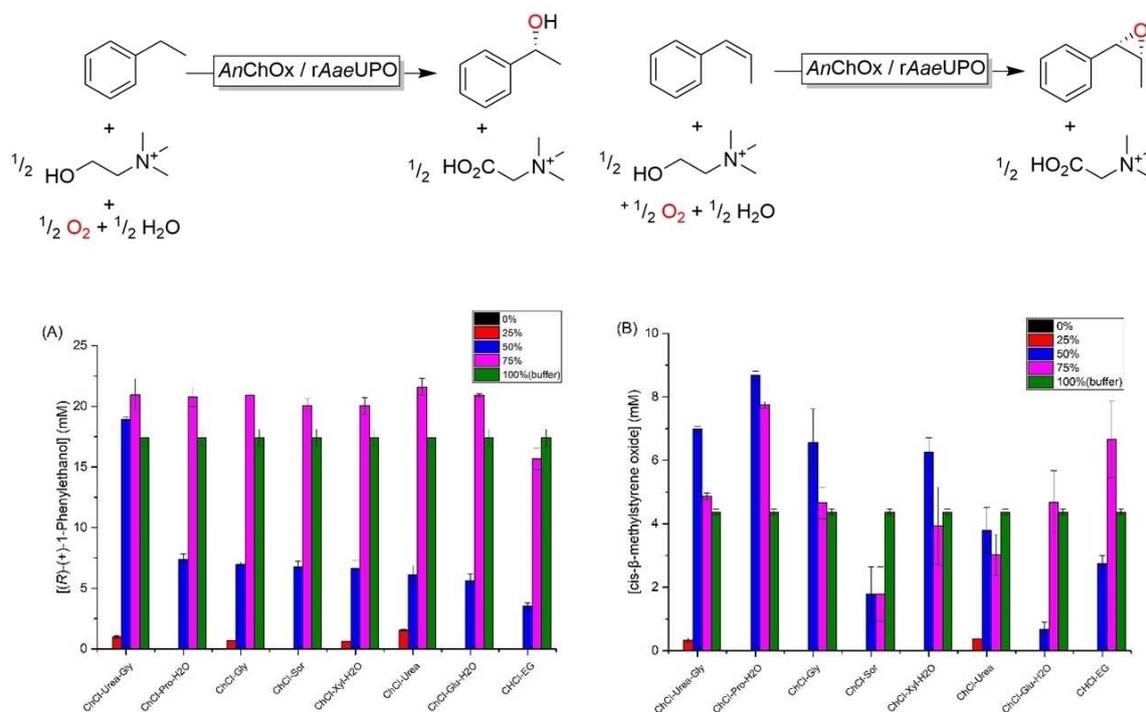
lished protocols.<sup>[9–10]</sup> The choline chloride (ChCl)-based NADES used in this study (Table S1) were synthesised as described previously.<sup>[8]</sup>

In a first set of experiments we evaluated the effect of the ChCl-based NADES on the bienzymatic hydroxylation of ethyl benzene and the epoxidation of *cis*- $\beta$ -methylstyrene (Figure 1). It should be mentioned here that no product formation was observed in negative controls leaving out either *rAaeUPO* or *AnChOx*. Furthermore, the optical purities of the products were always higher than 95% *ee*. We therefore concluded that the product formation reported in this contribution was indeed a result of the cascade shown in Scheme 1. Various dilutions of the individual NADES were evaluated.

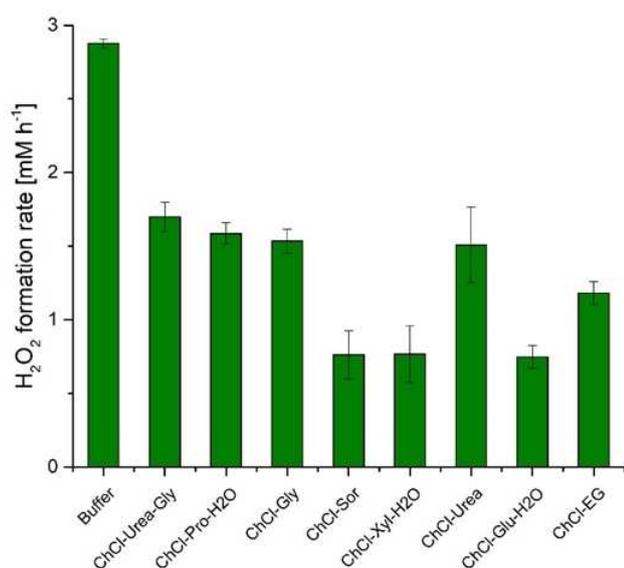
Noteworthy, neat NADES as solvents were unfavourable in all cases as no product formation was observed under these conditions, which at least partially can be attributed to the water-demand of the reaction (Scheme 1). For almost all NADES screened, at least one dilution with buffer was identified that enabled a higher product concentration than operating in aqueous buffer alone (the sole exceptions being the epoxidation reactions in ChCl-Sor and ChCl-EG). In case of the hydroxylation reaction, the highest product titers were observed in the presence of 25% NADES (Figure 1a, pink) while in case of the epoxidation reaction 50% was found more favourable (Figure 1b, blue). Apparently, for every reaction or substrate an optimal solvent composition exists. Currently, we are lacking a satisfactory explanation for this observation. Possibly, solubility issues play a role; a preliminary MD simulation suggested that some of the NADES can penetrate *rAaeUPO*'s active site (Figure S4 and S5). Hence, different NADES may influence the orientation of the substrates relative to compound I and thereby may influence *rAaeUPO*'s catalytic efficiency. More systematic studies will be necessary to fully elucidate this phenomenon and predict the optimal solvent for a given reaction.

Also, *AnChOx* activity was somewhat influenced by different NADES (Figure 2). Interestingly, *AnChOx* activity was generally reduced compared to the aqueous reaction medium.

Next, we investigated the influence of some NADES on the stability of *rAaeUPO* by incubating the biocatalyst in the respective NADES or in buffer under reaction conditions (Fig-



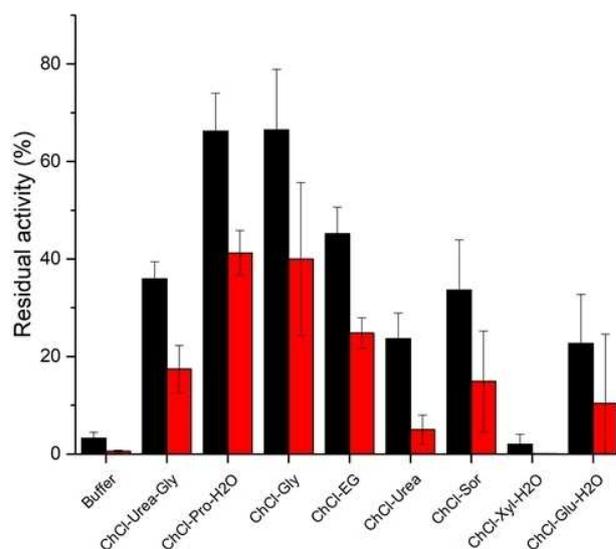
**Figure 1.** Evaluation of some NADES in varying dilutions for the bienzymatic hydroxylation of ethyl benzene (a) and the epoxidation of *cis*- $\beta$ -methylstyrene (b). Reaction condition: total reaction volume: 1 mL, [*AnChOx*] = 5  $\mu\text{M}$ , [*rAaeUPO*] = 100 nM, [Substrate] = 25 mM. NaPi buffer: pH 7.0, 50 mM, [ChCl] = 100 mM; NADES system: X % (v/v) DES was added to NaPi buffer (pH 7.0, 50 mM). Reaction mixtures were thermostated at 30 °C, stirred at 500 rpm for 24 h. Results represent the average of duplicates.



**Figure 2.** *AnChOx* activity in different NADES-buffer (50:50 v/v) mixtures. Reaction condition: 4 mL of NaPi buffer (pH 7.0, 50 mM) or NADES containing 5  $\mu$ M *AnChOx* was added to a transparent glass vial. In NaPi buffer (pH 7.0, 50 mM) system, [ChCl] = 100 mM, and in NADES system, 50% (v/v) NADES was added in NaPi buffer (pH 7.0, 50 mM). Reaction mixtures were thermostated at 30 °C, stirred at 500 rpm. Afterwards, the concentration of H<sub>2</sub>O<sub>2</sub> was determined by UV-Vis spectrophotometer using the following methods: 100  $\mu$ L of reaction mixtures were added in 1.5 mL tube with 100  $\mu$ L 5 mg L<sup>-1</sup> HRP and 1 mL 2 mM ABTS. The final conditions of this reaction were: [AnChOx] = 416.67 nM, [HRP] = 416.67 ng L<sup>-1</sup>, [ABTS] = 1.67 mM. The tube was thermostated at 25 °C for 10 min. The concentration of H<sub>2</sub>O<sub>2</sub> was calculated from the standard curve. Results represent the average of duplicates.

ure 3). Quite interestingly, while under reaction conditions, *rAaeUPO* exhibited poor robustness in the buffered system (the enzyme lost more than 90% of its initial activity within the 24 h), NADES (with the exception of ChCl-Xyl-H<sub>2</sub>O) had a very pronounced stabilising effect on the biocatalyst.

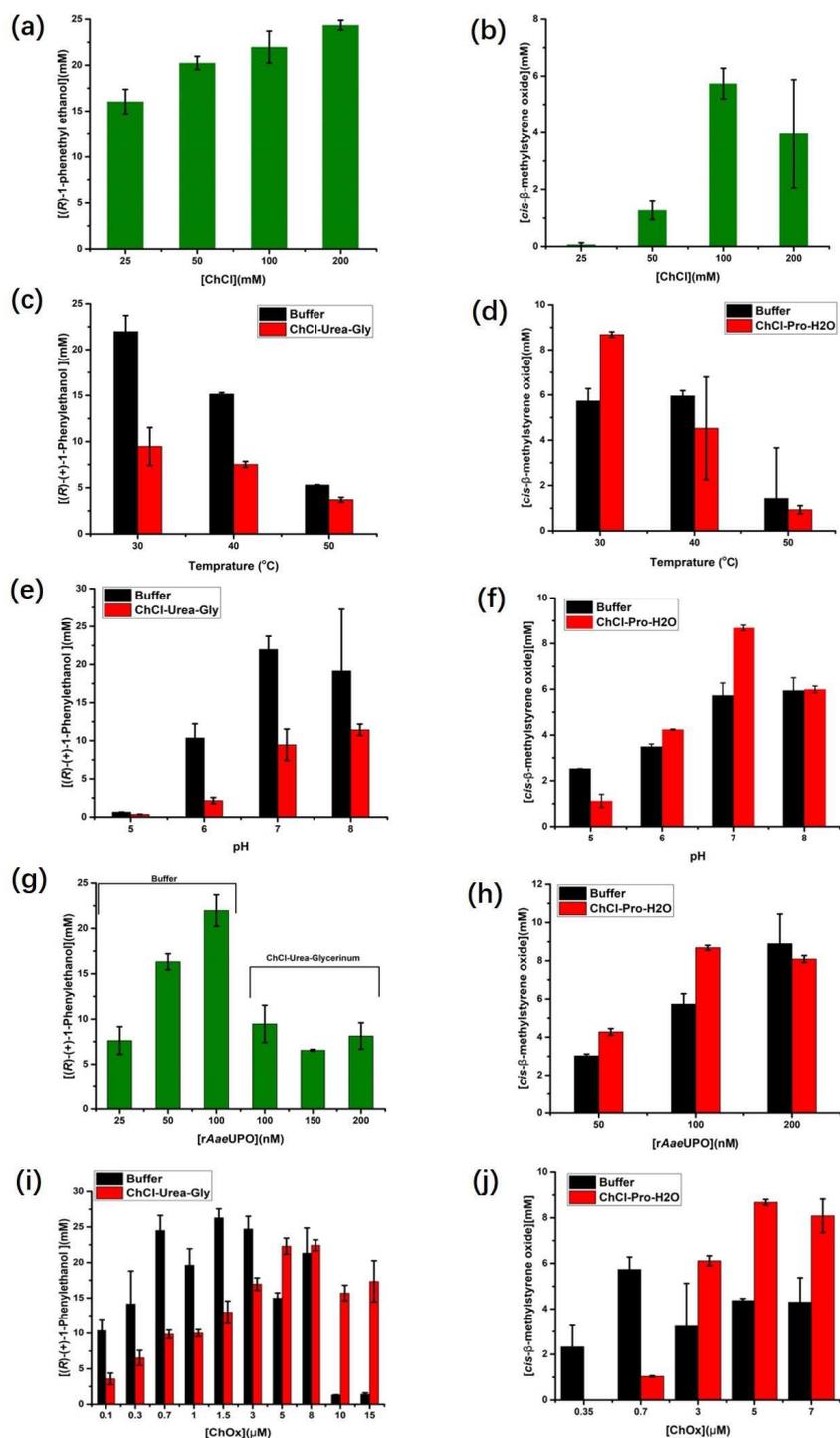
Based on these observations, we chose ChCl-Urea-Gly as solvent for the hydroxylation of ethyl benzene and ChCl-Pro-H<sub>2</sub>O for the epoxidation of *cis*- $\beta$ -methylstyrene, respectively. The influence of some additional reaction parameters such as the concentration of choline chloride (ChCl), the concentration of biocatalysts (*rAaeUPO* and *AnChOx*), reaction pH, reaction temperature in buffer system were investigated in some more detail (Figure 4). Again, some interesting differences between both reaction systems became apparent. In case of the hydroxylation reaction (in buffer) the product formation steadily increased over the concentration of ChCl range investigated. Contrarily, the epoxidation reaction (in buffer) had an apparent optimum at 100 mM ChCl (Figure 4a and 4b). One possible explanation for this difference may lie in the lower specific activity of *rAaeUPO* for the epoxidation reaction as compared to the hydroxylation reaction. It may be assumed that higher ChCl concentrations increased the H<sub>2</sub>O<sub>2</sub> generation rate. Hence, the optimal H<sub>2</sub>O<sub>2</sub> formation rate for the slower epoxidation reaction is reached at lower ChCl concentrations as compared to the faster hydroxylation reaction. This assumption is confirmed by



**Figure 3.** Residual activity of *rAaeUPO* after incubation under reaction conditions for 24 h (■) and 48 h (■). Incubation condition: in buffer system, the total volume was 1 mL, [rAaeUPO] = 100 nM, NaPi buffer (pH 7.0, 50 mM); in NADES system: the total volume is 1 mL, 30 °C, 500 rpm, [rAaeUPO] = 100 nM, added 50% (v/v) NaPi buffer (pH 7.0, 50 mM) into NADES. In all of them incubation was carried out at 30 °C, 500 rpm. ABTS tests reaction condition: Each time 20  $\mu$ L enzyme sample were transferred into 96-well plate, 30  $\mu$ L 2 mM ABTS were added, 20  $\mu$ L 20 mM H<sub>2</sub>O<sub>2</sub> were added, 100  $\mu$ L sodium citrate buffer (pH 4.0, 100 mM) were added, 30  $\mu$ L deionized water were added, and the absorbance was measured at 418 nm (The final concentration: [ABTS] = 0.3 mM, [H<sub>2</sub>O<sub>2</sub>] = 2 mM, [rAaeUPO] = 10 nM). Results represent the average of duplicates.

experiments systematically increasing the concentration of *rAaeUPO* (Figure 4g and 4h) and *AnChOx* (Figure 4i and 4j). It is interesting to note that the NADES systems supported higher ChCl and *AnChOx* concentrations as compared to the buffer systems. This may indicate a protective effect of NADES on the enzymes against H<sub>2</sub>O<sub>2</sub>. Similar observations had been previously reported.<sup>[11]</sup> Also, the temperature-dependence of both reactions was very different. As shown in Figures 4c and 4d, the apparent activity of the hydroxylation reaction in NADES decreased by only 50% while raising the reaction temperature from 30 °C to 50 °C (the same reaction in buffer medium decreased by more than 75%). However, the apparent activity of the epoxidation rate decreased by more than 80% in NADES. Possibly, epoxide hydrolysis or isomerisation at elevated temperatures may account for this observation. A close inspection of the gas chromatograms gave no indication of other products than the desired epoxide. Currently, we are lacking a plausible explanation for this observation.

In the present study we have demonstrated that Choline oxidase is a very promising catalyst for the *in situ* generation of H<sub>2</sub>O<sub>2</sub> to drive peroxygenase-catalysed hydroxylation and epoxidation reactions. This system is particularly interesting when combining with ChCl-based NADES as here the solvent serves two purposes at the same time, i.e. as solvent (and performance additive stabilising the biocatalysts) and as cosubstrate providing the reducing equivalents needed for the reductive activation of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>.



**Figure 4.** Influence of reaction parameters such as concentration of ChCl in buffer system (a, b), reaction temperature (c, d), pH (e, f), concentration of *rAaeUPO* (g, h) and concentration of *AnChOx* (i, j) on the rate of the bienzymatic hydroxylation of ethyl benzene (a, c, e, g, i) and *cis*-β-methylstyrene (b, d, f, h, j). Experimental details are provided in the Supporting Information. Results represent the average of duplicates.

Some of the results obtained in this study still need further investigation. Particularly the question remains why the type of reaction (or substrate) has an influence on the optimal reaction conditions (choice of NADES). Also the effect of viscosity on the reaction kinetics deserve further, in-depth investigation.

It should, however, also be emphasised that, despite the many open questions, the catalytic performance of the current system is already excellent. Under optimised conditions, *rAaeUPO* performed more than 200000 (Table S3) and more than 80000 (Table S6) catalytic turnovers in the hydroxylation and epoxidation reaction, respectively.

## Experimental Section

### Chemical reagents and materials

In this work, all chemicals were purchased from Sigma-Aldrich, TCI or Aladdin with the highest purity available and used without further treatment. Water was purified with a Millipore (Bedford, MA) Milli-Q water system.

### Preparation of AnChOx

Proteins of AnChOx was expressed in *E.coli* BL21 (DE3) gold cells (Weidibio, Shanghai, China). Precultures of *E.coli* BL21 (DE3) gold cells in 25 mL LB-media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 mg/L kanamycin) were incubated overnight (12 h, 37°C, 200 rpm) and used to inoculate the main cultures. The main cultures (500 mL LB-media) were mixed with the inoculum until an optical density of 0.01 was reached. Main cultures were cultivated until an OD<sub>600 nm</sub> of 0.8 (4 h, 37°C, 200 rpm) could be observed. Protein overexpression was induced by supplementing isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.05 mM final concentration). Upon induction, the cultivation temperature was reduced to 20°C. Cells were harvested after 5 h by centrifugation (4000 rpm, 20 min, 4°C). The obtained cell pellets were suspended in 20 mM sodium phosphate buffer, 500 mM NaCl, pH 7.5, and disrupted by sonication on ice. Soluble proteins were separated from cell fragments and insoluble proteins by centrifugation (10000 rpm, 40 min, 4°C). The supernatant was filtered through a 0.45 μm cellulose-acetate filter and subsequently used for further purification.

### Purification of AnChOx

The AnChOx proteins were purified via chromatography and dialysis (supplementary material). Protein concentrations were determined with the BCA protein assay kit and protein homogeneity was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### Preparation of ChCl-based NADES

ChCl-based NADES can be easily prepared either by thermally treating the admixed precursors. Following a published method,<sup>[5,8b]</sup> choline chloride was mixed with others at an appropriate molar ratio at 80°C under rigorous agitation for 2 h continuous stirring until colourless, homogeneous liquids were obtained. Different ChCl-based NADES molar ratios as given in the parentheses: ChCl-Sor: choline chloride-sorbitol (1:1), ChCl-Gly: choline chloride-glycerol (1:2), ChCl-EG: choline chloride-ethylene glycol (1:1), ChCl-Urea: choline chloride-urea (1:2), ChCl-Urea-Gly: choline chloride-urea-glycerol (1:1:1), ChCl-Xyl-H<sub>2</sub>O: choline chloride-xylopyranose-H<sub>2</sub>O (5:2:5), ChCl-Pro-H<sub>2</sub>O: choline chloride-1,2-propanediol-H<sub>2</sub>O (1:1:1), ChCl-Glu-H<sub>2</sub>O: choline chloride-glucose-H<sub>2</sub>O (1:1:3).

### Enzymatic reactions using rAaeUPO

Enzymatic reactions using rAaeUPO were performed at 30°C, 500 rpm in 1.0 mL of NaPi buffer (pH 7.0, 50 mM) or NADES. All reactions were carried out in a 5 mL glass vials submerged in a thermostatic oil bath for temperature control. Unless mentioned otherwise, firstly, 0.5 mL of NADES and 0.3 mL NaPi buffer (pH 7.0, 50 mM) were added into the reaction vessel, then 25 mM of substrates (ethylbenzene or cis-β-methylstyrene or phenyl vinyl sulfide), 100 nM rAaeUPO and 5 μM of AnChOx were added into the reaction vessel (final concentration). The volume of the reaction

mixture was adjusted to 1.0 mL in a final step. The reaction vial was closed. After the reaction is over, substrate and products were extracted with ethyl acetate (containing 50 mM of dodecane as internal reference) and analysed by gas chromatography.

### Details of gas chromatography and temperature profiles

An Agilent 7890B GC system (Agilent Technologies, Palo Alto, CA, USA) was used in the study. Chromatographic separation was conducted by a Agilent J&W CP-Sil 88 (Agilent Technologies, Palo Alto, CA, USA) GC column (60 m length × 0.25 mm I.D. × 0.20 μm film thickness) and a Agilent J&W CP-Chirasil-Dex CB (Agilent Technologies, Palo Alto, CA, USA) GC column (25 m length × 0.32 mm I.D. × 0.25 μm film thickness) were used for achieving chromatographic separation. The injector temperature, 250°C; split mode (30:1); detector temperature 280°C; GC oven temperature for working solutions and samples and retention times of intermediates and GC standard are listed in Supporting Information.

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** Peroxygenases · Deep Eutectic Solvents · Choline oxidase · Oxyfunctionalisation reactions

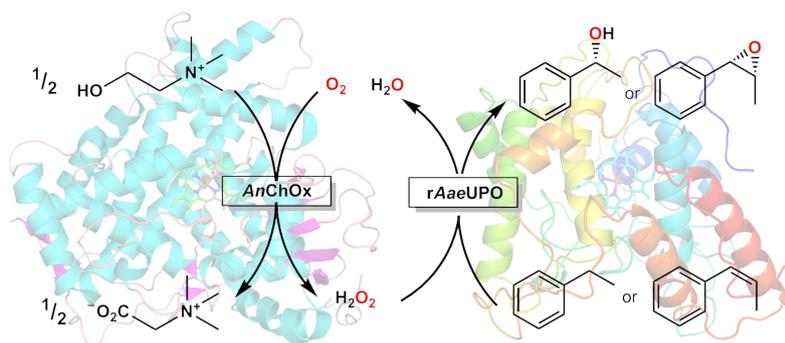
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## COMMUNICATIONS



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from *Agrocybe aegerita* (rAaeUPO) and choline oxidase from *Arthrobacter nicotianae* (AnChOx) to provide rAaeUPO with H<sub>2</sub>O<sub>2</sub>.