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Enzymatic Oxidation of Butane to 2-Butanol in a Bubble Column

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Unspecific peroxygenases have recently gained significant interest due to their ability to catalyse the hydroxylation of non-activated C–H bonds using only hydrogen peroxide as a co-substrate. However, the development of preparative processes has so far mostly concentrated on benzyl hydroxylations using liquid substrates. Herein, we demonstrate the application of a peroxygenase for the hydroxylation of the inert, gaseous substrate butane to 2-butanol in a bubble column reactor. The influence of hydrogen peroxide feed rate and enzyme loading on product formation, overoxidation to butanone and catalytic efficiency is investigated at 200 mL scale. The process is scaled up to 2 L and coupled with continuous extraction. This setup allowed the production of 115 mmol 2-butanol and 70 mmol butanone with an overall total turnover number (TTN) of over 15,000, thereby demonstrating the applicability of peroxygenases for preparative hydroxylation of such inert, gaseous substrates at mild reaction conditions.

Butane is produced at kt per year scale as a side product in the oil refinery industry.[1] The inertness of butane precludes its use as feedstock for the synthesis of value-added products. Instead, it is used for thermal applications; in other words, it is burned to create heat. Chemical technologies to introduce functional groups such as hydroxy- or C–C-groups are poorly developed and suffer from harsh reaction conditions as well as poor selectivity.[2]

Monoxygenases are promising alternative catalysts that circumvent the above-mentioned limitations.[3,4] Particularly (non-) heme iron monoxygenases are powerful enzymes for the selective oxyfunctionalisation of non-activated C–H-bonds. The complicated molecular architecture of monoxygenases, however, largely limits their application to whole cell systems. More recently, so-called unspecific peroxygenases (E.C. 1.11.2.1) are gaining interest as catalysts for selective oxyfunctionalisation chemistry.[5,6] Like the prevalent P450 monoxygenases, peroxygenases convert a broad range of starting materials but rely only on H2O2 as stoichiometric co-substrate. The peroxygenase from the fungus Agrocybe aegerita (AaeUPO)[7] catalyses the sub-terminal hydroxylation of a broad range of fatty acids[8] and alkanes[9] and therefore appears to be a promising catalyst for the selective transformation of butane to 2-butanol (Scheme 1), which was first presented in analytical scale.[9]
This reaction has been demonstrated before using P450s and while impressive turnover frequencies up to 30.5 s\(^{-1}\) were achieved,\(^{[24,10]}\) product concentrations remained below preparative scale and no data concerning catalyst efficiency in terms of TTN has been reported.\(^{[4,11]}\) In this study, we utilised the recombinant, evolved variant (rAaeUPO).\(^{[2,22]}\)

Another advantage of H\(_2\)O\(_2\)-driven hydroxylation reactions is that gaseous O\(_2\) can be avoided. As a result, the explosion hazard is reduced and pure butane (instead of butane/inert gas mixtures) can be fed to the reactor. This is also expected to maximise the phase transfer rate of butane into the aqueous, enzyme-containing reaction medium. Moreover, surplus butane remains undiluted throughout the process, which allows direct recycling of the off-gas. Nonetheless, the physical properties of the starting material (particularly, its high volatility and poor water solubility), pose a significant challenge to the practical implementation of the envisioned rAaeUPO-catalysed hydroxylation reaction. To achieve sufficient mass transfer of the gaseous substrate, a bubble column reactor was used in this study. This reactor setup offers a number of advantages for the reaction investigated here: it allows sufficient mixing at a low power input and hence minimises the shear stress on the biocatalyst. Furthermore, the absence of moving parts minimises safety issues when operating with flammable gases.

To determine the catalyst efficiency,\(^{[15]}\), which is also strongly influenced by hydrogen peroxide feeding,\(^{[16]}\) we set out to investigate this UPO-catalysed butane oxidation at varying reaction conditions.

As a starting point for our investigations, we used an enzyme concentration of 1.4 \(\mu\)M with a butane feed of 7.5 Lh\(^{-1}\) and a H\(_2\)O\(_2\) feed of 1 mMh\(^{-1}\) in a 0.25 L bubble column (0.5vvm, see SI). Under these conditions, linear product formation was observed for at least 2.5 h (Figure S3). Interestingly, the product formation rate (approx. 0.5 mMh\(^{-1}\)) was only half of the theoretical rate determined by the H\(_2\)O\(_2\) feed rate. To obtain further insights into the influence of the H\(_2\)O\(_2\) feeding rate we performed an experiment gradually increasing the H\(_2\)O\(_2\)-feeding rate (—), butanone n.d.. At intervals, samples were taken from the reaction setup to determine the residual rAaeUPO activity.

Increasing the concentration of all reagents (i.e. 4 × rAaeUPO concentration, 15 × H\(_2\)O\(_2\) feed rate) resulted in a drastically increased butane hydroxylation rate (Figure S5) leading to more than 30 mM of 2-butanol and a productivity of 13.3 mMh\(^{-1}\). This, however, also came along with a decreased robustness of the biocatalyst being fully inactivated within less than 3 h (TTN = 5710). Possibly, this is the result of ‘hot spots’ of high H\(_2\)O\(_2\) concentration at the feed inlet (the H\(_2\)O\(_2\) concentration in the feed solution was almost 9 times higher than in the experiment shown in Figure 2). It is also interesting to note that in this experiment, possibly because of the overall higher 2-butanol concentration, a significant further oxidation to butanone (accounting for approx. 15% of the overall product) was observed.

A comparison of yields concerning hydrogen peroxide (Figure S9) showed that the co-substrate was utilised sub-stoichiometrically with decreasing efficiency at higher feed rates. The overall low yield might be partially explained by the unspecific oxidation of fermentation residues that were introduced with the crude enzyme.
Moreover, butanol evaporation could have lowered the yield to some extent. The decrease in yield at higher \( \text{H}_\text{2} \text{O}_\text{2} \) feed rates might be attributable to the catalase reaction as local concentration maxima would be more pronounced under these conditions.

To demonstrate the feasibility of a preparative synthesis in this system, we sought to increase the scale to 2 L while maintaining high productivity conditions. In this experiment, we also decided to apply an \textit{in situ} product removal system (see Figure 3) to facilitate product isolation in a later preparative scale setup.

Due to the high activity of \textit{rAaeUPO} with a broad range of organic solvents, possibly resulting in undesired hydroxylation of the organic phase, we decided to decouple the reactive and extractive reactor parts by coupling the bubble column to a second, extractive column, in which no hydrogen peroxide was supposed to be present to prevent extractant oxidation. Requirements for the extractant were water solubility lower than that of butane and a suitable partition coefficient for 2-butanol. \textit{n-decanol} was chosen due to its good selectivity for 2-butanol (\( P_{\text{n-decanol/\text{H}_2\text{O}}} = 3.2 \)) and its low water solubility (0.25 mM)\([15]\) compared to butane (\( > 1 \text{ mM} \))\([16]\).

Enzyme concentration and hydrogen peroxide feed rate were maintained from the previous experiment while the butane feed rate was scaled based on maintaining a constant superficial gas velocity (Figure 4). With this setup, similar aqueous concentrations of 2-butanol were produced, while a significant amount of 2-butanol and butanone were extracted by the organic phase. The overall amount of product was nearly doubled as compared to the previous experiment (Table S4) also resulting in a drastically increased TTN (more than 16000) of the biocatalyst. This TTN is well in line with other aliphatic, saturated C-H hydroxylation catalysed by UPOs\([17]\).

The \textit{rAaeUPO}-catalysed overoxidation of 2-butanol to butanone was more pronounced in this experiment (38% of the total product), which may be the result of a decreased specific gassing rate as compared to the previous experiment and the resulting higher abundance of 2-butanol for the peroxidase reaction.

Using online hydrogen peroxide monitoring, we could show that reaction medium entering the extraction column contained almost no more \( \text{H}_\text{2} \text{O}_\text{2} \), thereby minimising the possibility of undesired oxidation of the organic phase (\textit{n-decanol}). 2-butanol accumulated in the organic phase up to a partition coefficient of 2.4, while butanone showed equal partitioning between aqueous and organic phase. Overall, 8.5 g of 2-butanol and 5.6 g of butanone were obtained in this experiment.

In this contribution we have demonstrated that selective functionalisation of inert butane is possible using peroxygenases. A comparison of the catalytic performances of \textit{rAaeUPO} in the different reaction setups (Table 1) shows high optimisation potential of this process.

One point of attention is the comparably low robustness of the biocatalysts. While \textit{rAaeUPO} is intrinsically robust and can stay active under operational conditions for days, here \textit{rAaeUPO} lost its activity within 3–6 h\([18]\). Possibly, the demanding reaction conditions caused by aeration in the bubble column are partially responsible for this. Also the \( \text{H}_\text{2} \text{O}_\text{2} \) supply method, generating ‘hot spots’ where the
enzyme is exposed with locally very high $\text{H}_2\text{O}_2$ concentrations leading to rapid inactivation\cite{20}, contributes. Immobilisation of the enzyme\cite{21}, which has been demonstrated at pilot scale for bubble columns reactors,\cite{22} will be evaluated to stabilise it. Also, in situ generation of $\text{H}_2\text{O}_2$ will avoid concentration gradients within the reactor and by this means will increase the catalyst efficiency. In the context of the proposed reaction, the use of a gas diffusion electrode will be especially interesting, as this method does not require molecular oxygen to be dissolved in the bulk medium, is therefore not detrimental to process safety and has been proven to be compatible with UPO catalysed processes.\cite{23,24} The use of in situ production should also help to gain a better understanding of the effect of co-substrate supply on the $\text{H}_2\text{O}_2$ yield.

A second challenge that became apparent in this work was product overoxidation. Future work will therefore evaluate the use of more efficient product removal systems, such as more efficient extractants, countercurrent extraction and membrane-based methods, which should help with the complete removal of product from the reaction system. Moreover, detailed kinetic investigations concerning overoxidation will be carried out and the use of evolved UPO mutants that exhibit lower oxidation activity\cite{25} will be evaluated.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords:** bubble column · butane · butanol · hydroxylation · peroxygenase

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
& Low enzyme & High enzyme & Extractive scale-up \\
\hline
\text{(Figure 2)} & & & \\
\hline
TTON [s$^{-1}$] & 6516 & 5710 & 16290 \\
\hline
TOF [s$^{-1}$] & 0.30 & 0.53 & 1.13 \\
\hline
\end{tabular}
\caption{Comparison of catalytic performance of rNaeUPO in the oxyfunctionalisation of butane. Calculations for hydroxylation performance are based on the sum of butane molecules hydroxylated (i.e. 2-butanol + butanone). Catalytic parameters concerning hydroxylation and oxidation reactions separately, see Table S4.}
\end{table}

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**Enzymatic oxidation:** To date the selective oxyfunctionalisation of chemically inert short chain alkanes pose a challenge for bio- and chemo-catalysis. In this work we present the hydroxylation of butane using peroxigenases from *Agrocybe aegerita* in a 0.2 and 2 L bubble column reactor setup.

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1 – 5