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DOI
10.1002/cctc.202000431

Publication date
2020

Document Version
Final published version

Published in
ChemCatChem

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

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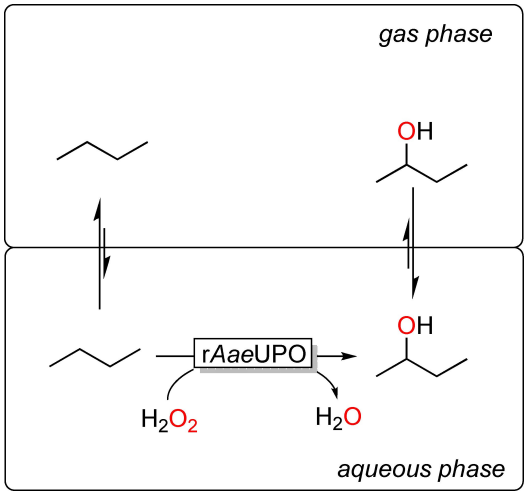
Unspecific peroxygenases have recently gained significant interest due to their ability to catalyse the hydroxylation of non-activated C–H bonds using only hydrogen peroxide as a co-substrate. However, the development of preparative processes has so far mostly concentrated on benzylic hydroxylations 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 hydroxyl- or C=C-groups are poorly developed and suffer from harsh reaction conditions as well as poor selectivity.[2]

Monooxygenases are promising alternative catalysts that circumvent the above-mentioned limitations.[3,4] Particularly (non-) heme iron monooxygenases are powerful enzymes for the selective oxyfunctionalisation of non-activated C–H-bonds. The complicated molecular architecture of monooxygenases, 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 monooxygenases, peroxygenases convert a broad range of starting materials but rely only on H_2O_2 as stoichiometric co-substrate. The peroxygenase from the fungus *Agrocybe aegerita* (*Aae*UPO)[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]



Scheme 1. Envisioned hydroxylation of butane to 2-butanol in a two-phase reaction setup using butane as gaseous phase. r*Aae*UPO: recombinant, evolved peroxygenase from *Agrocybe aegerita*.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.202000431

This reaction has been demonstrated before using P450s and while impressive turnover frequencies up to 30.5 s^{-1} where achieved,[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 (r*Aae*UPO).[12]

Another advantage of H_2O_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 r*Aae*UPO-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[13], which is also strongly influenced by hydrogen peroxide feeding,[14] 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 μM with a butane feed of 7.5 L h^{-1} and a H_2O_2 feed of 1 mM h^{-1} in a 0.25 L bubble column (0.5 vvm, 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 mM h^{-1}) was only half of the theoretical rate determined by the H_2O_2 feed rate. To obtain further insights into the influence of the H_2O_2 feeding rate we performed an experiment gradually increasing the H_2O_2 dosing rate (Figure 1). Upon stepwise increasing the H_2O_2 feed from 1 mM h^{-1} to 16 mM h^{-1}, a proportional increase of the 2-butanol productivity was observed. As a consequence, the catalytic activity of r*Aae*UPO in terms of turnover frequency (TOF = moles of product divided by the moles of r*Aae*UPO per time) increased from 0.1 s^{-1} to more than 2 s^{-1} (Figures S4). It is also interesting to note that up to a H_2O_2 feed rate of 8 mM h^{-1}, the enzyme activity under operational conditions was almost constant. Increasing the H_2O_2 feed rate further to 16 mM h^{-1} resulted in a rapid inactivation of the biocatalyst.

To better assess the kinetics of enzyme inactivation, a constant hydrogen peroxide feeding rate of 4 mM h^{-1} was chosen, while enzyme concentration and butane feed rate of the previous experiment were maintained (Figure 2). This resulted in the production of 9.5 mM 2-butanol and 0.9 mM butanone, the over-oxidation product of 2-butanol, over the course of 6 h. The measured TTN in this experiment was approximately 6500. The hydrogen peroxide concentration, measured online in a bypass of the bubble column reactor, remained below 0.1 mM throughout the course of the reaction and only increased after 5.5 h when the enzyme was mostly inactivated (Figure S6).

Increasing the concentration of all reagents (i.e. 4 × r*Aae*UPO concentration, 15 × H_2O_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 mM h^{-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_2O_2 concentration at the feed inlet (the H_2O_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.

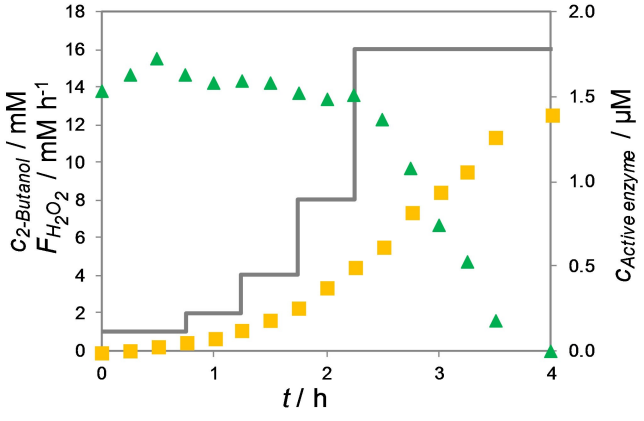

Figure 1. r*Aae*UPO-catalysed hydroxylation of butane at increasing H_2O_2 feeding rates: Active enzyme (▲), 2-butanol (■), H_2O_2-feeding rate (—), butanone n.d.. Reaction conditions: 25 °C, butane 21 L h^{-1}, 200 mL initial volume, H_2O_2-feed 100 mM (1–8 mM h^{-1}), 200 mM (16 mM h^{-1}). At intervals, samples were taken from the reaction setup to determine the residual r*Aae*UPO activity.

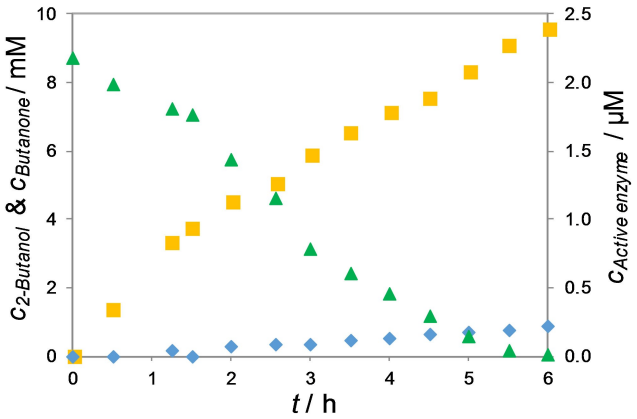

Figure 2. r*Aae*UPO-catalysed hydroxylation of butane at a constant H_2O_2 feeding rate of 4 mM h^{-1}: Active enzyme (▲), 2-butanol (■), butanone (♦). Reaction conditions: 25 °C, butane 21 L h^{-1}, 200 mL initial volume, H_2O_2-feed 100 mM, 8 mL h^{-1}. Unspecific overoxidation by H_2O_2 was not observed (*cf.* Figure S10).

Moreover, butanol evaporation could have lowered the yield to some extent. The decrease in yield at higher H_2O_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 *in situ* product removal system (see Figure 3) to facilitate product isolation in a later preparative scale setup.

Due to the high activity of r*Aae*UPO 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. n-decanol was chosen due to its good selectivity for 2-butanol ($P_{decanol/H2O} = 3.2$) and its low water solubility (0.25 mM)[15] compared to butane (> 1 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 hydroxylations catalysed by UPOs.[17]

The 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.

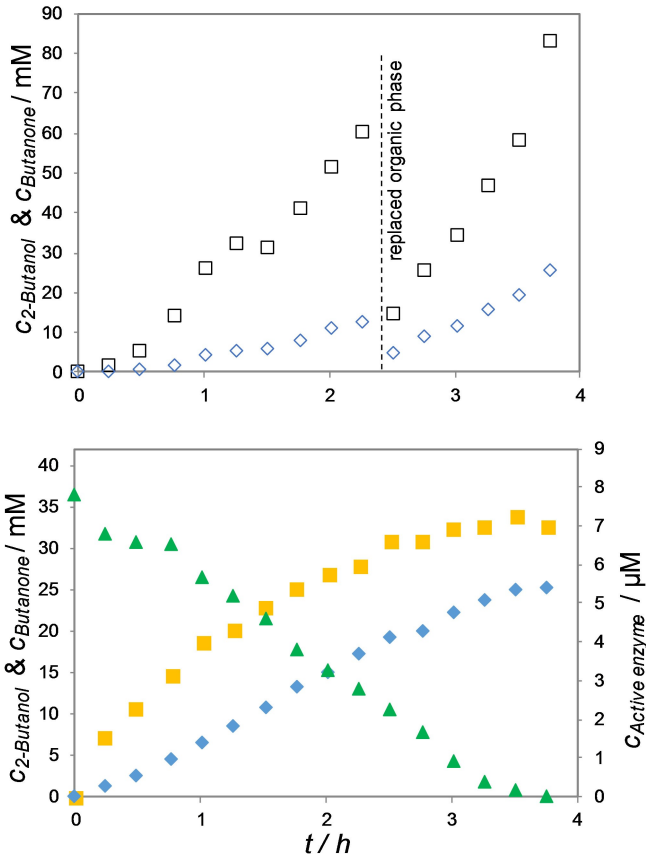

Figure 4. Scale-up and coupling of r*Aae*UPO-catalysed hydroxylation of butane with extraction at high enzyme loading and a constant H_2O_2 feeding rate of 60 mM h^{-1}: Aqueous phase (lower graph): Active enzyme (▲), 2-butanol (■), butanone (♦); Organic phase (upper graph): 2-butanol (□), butanone (◇). Reaction conditions: 25 °C, butane 61.4 L h^{-1}, 2000 mL initial volume aqueous phase, 200 mL n-decanol, H_2O_2-feed 882 mM, 136 mL h^{-1}. The organic phase was replaced with 200 mL fresh *n*-decanol after 2.4 h.

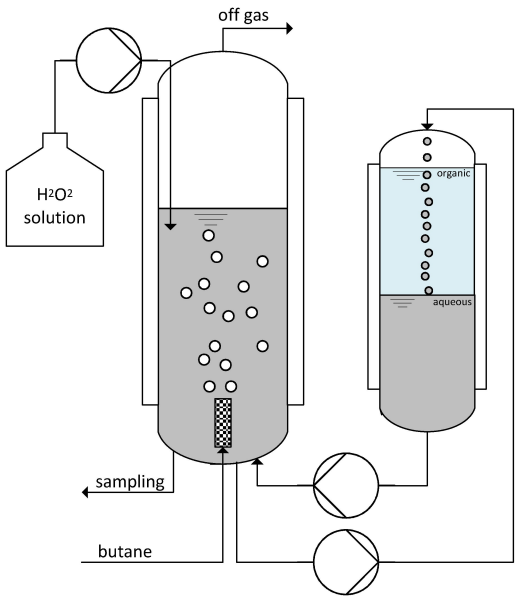

Figure 3. Experimental setup for the upscaled bubble column reactor (middle) coupled with an extractive column (right).

Using online hydrogen peroxide monitoring, we could show that reaction medium entering the extraction column contained almost no more H_2O_2, thereby minimising the possibility of undesired oxidation of the organic phase (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 r*Aae*UPO 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 r*Aae*UPO is intrinsically robust and can stay active under operational conditions for days, here r*Aae*UPO 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 H_2O_2 supply method, generating 'hot spots' where the

Table 1. Comparison of catalytic performance of rAaeUPO 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.

	Low enzyme (Figure 2)	High enzyme (Figure S5)	Extractive scale-up (Figure 4)
TTN	6516	5710	16290
TOF [s^{-1}]	0.30	0.53	1.13

enzyme is exposed with locally very high H_2O_2 concentrations leading to rapid inactivation[19], contributes. Immobilisation of the enzyme[20], which has been demonstrated at pilot scale for bubble columns reactors,[21] will be evaluated to stabilise it. Also, *in situ* generation of H_2O_2[6] 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.[14,22] The use of *in situ* production should also help to gain a better understanding of the effect of co-substrate supply on the H_2O_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[23] will be evaluated.

Acknowledgements

F.H. gratefully acknowledges funding by the European Research Commission (ERC consolidator grant, No. 648026) and the Netherlands Organization for Scientific Research (VICI grant, No. 724.014.003).

Conflict of Interest

The authors declare no conflict of interest.

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

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Manuscript received: March 11, 2020
Revised manuscript received: April 26, 2020
Accepted manuscript online: April 28, 2020
Version of record online: ■■, ■■■

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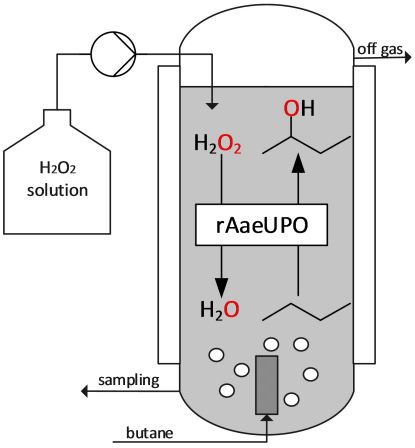
Enzymatic oxidation: To date the selective oxyfunctionalisation of chemically inert short chain alkanes pose a challenge for bio- and chemocatalysis. In this work we present the hydroxylation of butane using peroxygenases from *Agrocybe aegerita* in a 0.2 and 2 L bubble column reactor setup.



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