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Efficient Aerobic Oxidation of *trans*-2-Hexen-1-ol using the Aryl Alcohol Oxidase from *Pleurotus eryngii*

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Abstract: The selective oxidation of *trans*-2-hexen-1-ol to the corresponding aldehyde using a recombinant aryl alcohol oxidase from *Pleurotus eryngii* (PeAAOx) is reported. Especially using the two liquid phase system to overcome solubility and product inhibition issues enabled to achieve more than 2.200.000 catalytic turnovers for the production enzyme as well as molar product concentrations, pointing towards an economic feasible reaction.

Keywords: biocatalysis; oxidase; two liquid phase system; oxidation reactions

The selective oxidation of functionalised alcohols to the corresponding aldehydes still poses some challenges in synthetic organic chemistry.^[1] Issues with functional group tolerance, overoxidation and other undesired side reactions are still observed frequently.^[2] Also, traditional chemical routes are sometimes plagued by a rather high energy demands and dependence on environmentally questionable oxidants.^[3] When it comes to selectivity, enzymes are generally amongst the first catalysts to be mentioned.^[4] Nevertheless, only a few synthetic oxidation reactions rely on biocatalysis, and preference usually is given to the well-established homogeneous and heterogeneous catalysts. Partially, this is due to the (perceived) high

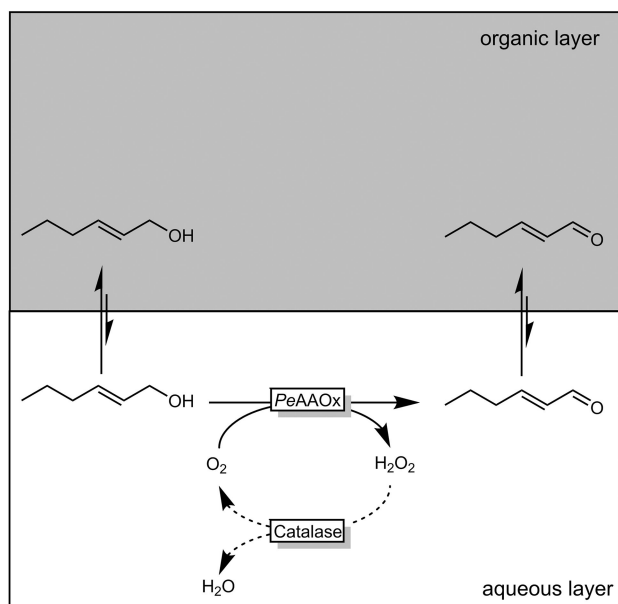
costs of enzyme production. Furthermore, product titres reported for biocatalytic oxidations still tend to be in the lower millimolar range (few g l^{-1}) and thus are unattractive from a preparative point-of-view.

To address these issues, we chose the oxidation of *trans*-2-hexen-1-ol to the corresponding aldehyde as model reaction. The corresponding aldehyde is widely used in the flavour and fragrance industry as fresh flavour ingredient (Green Note).^[5]

For the oxidation of alcohols a range of enzyme systems are available^[4c] with alcohol dehydrogenases (ADHs)^[4b,6] and alcohol oxidases (AOx) being the most useful ones.^[7] On the one hand, ADHs catalyse reversible, Meerwein-Ponndorf-Verley-like oxidation reactions, which generally necessitate high molar surpluses of the sacrificial oxidant (mostly acetone) to drive the equilibrium reaction. Alcohol oxidases, on the other hand, utilise molecular oxygen as oxidant, yielding H_2O_2 as stoichiometric by-product in an irreversible fashion. Hazardous hydrogen peroxide can easily be dismutated using catalase. Hence, AOx-catalysed oxidations appear more suitable from an environmental point-of-view (i.e. yielding less waste products) as compared to ADH-catalysed ones. Therefore, we drew our attention to the aryl alcohol oxidase from *Pleurotus eryngii* (PeAAOx).^[8]

The enzyme was heterologously expressed in *Escherichia coli*, *in vitro* reactivated and purified (see SI for a detailed description).

Both, the substrate and product of the reaction are sparingly soluble in aqueous reaction mixtures (130 and 60 mM in the reaction buffer used here, respectively). Therefore we evaluated the so-called two-liquid-phase-system approach (2LPS, Scheme 1). Here, a hydrophobic organic phase serves both, as substrate reservoir and product sink enabling overall high reagent loadings as demonstrated previously for various reactions.^[9]



Scheme 1. Biocatalytic oxidation of *trans*-2-hexen-1-ol. To achieve overall high reagent loadings, a hydrophobic organic phase is added to the aqueous reaction buffer. *PeAAOx*: aryl alcohol oxidase from *Pleurotus eryngii*. In order to alleviate the potentially harmful effect of H_2O_2 , catalase is added to the reaction.

The 2LPS also contributes to minimise enzyme inhibition by the product and undesired side reactions of the aldehyde in the aqueous phase.

In a first set of experiments we further elucidated the operational window for *PeAAOx* in terms of optimal pH and temperature and mechanical and solvent stability. Regarding the optimal pH, *PeAAOx* is active in a broad pH range, displaying the highest activity between pH 5 and 8 (Figure S1). Based on these results, pH 7 was selected for further experiments due to its compatibility with the activity of catalase (required for the dismutation of H_2O_2). *PeAAOx* exhibits the maximum activity at 30 °C with a turnover frequency of 25 s^{-1} . Above this temperature the activity dropped dramatically, with a 25 fold decrease at 40 °C ($\text{TF} < 1 \text{ s}^{-1}$, Figure S2). The decrease of the activity at elevated temperatures is most likely attributed to thermal denaturation of the biocatalyst.

We therefore conducted all further experiments at 20 °C as a compromise between high activity and stability. 2LPSs are frequently plagued by diffusion limitations over the phase border, which can be addressed by e.g. vigorous mixing to increase the surface area. We therefore investigated the robustness of *PeAAOx* against mechanical stress (Figure S3). Pleasingly, the enzyme was seemingly not affected by high shaking velocities.

Finally, also the stability of *PeAAOx* in the presence of various organic solvents was determined (Figure S4). Hydrophobic solvents such as isoctane or dodecane were tolerated well by the enzyme and initial rates up to 13 turnovers per second were achieved. Using toluene gave no catalytic conversion at all. Possibly, π -stacking interactions of the aromatic ring with the flavin prosthetic group resulted in a strong competitive inhibition of *PeAAOx*.^[8b] Quite surprisingly, even ethyl acetate was tolerated by *PeAAOx* as organic phase even though reactions ceased rather quickly due to acidification of the aqueous layer caused by autohydrolysis.

We chose dodecane as organic phase. Figure 1 shows a representative time course of a reaction in the biphasic reaction system. Pleasingly, full conversion of the starting material into the desired product (49 g l^{-1} organic phase) was observed within 24 h. The nominal catalytic performance of *PeAAOx* in the biphasic

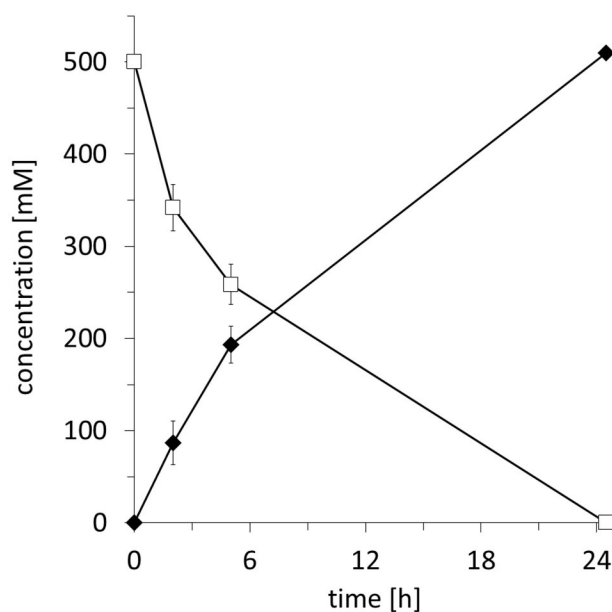


Figure 1. *PeAAOx*-catalysed oxidation of *trans*-2-hexen-1-ol (□) to *trans*-2-hexenal (◆) using a biphasic (2LPS) reaction system. Conditions: $T = 20\text{ }^\circ\text{C}$, shaking rate = 1000 rpm; aqueous phase: 0.5 ml of 50 mM KPi (pH 7), [*PeAAOx*] = 0.75 μM , [Catalase] = 720 U ml^{-1} (0.1 μM); organic phase: 0.5 ml of dodecane, [*trans*-2-hexen-1-ol] = 500 mM; phase ratio: 1:1 (v/v).

system (TF of 14.3 s^{-1} within the first 5 h) was somewhat lower compared to the monophasic reaction (TF of 22.8 s^{-1} , Figure S2), which most likely is attributed to phase transfer limitations of substrates (*trans*-hex-2-en-1-ol and O_2).^[10] Comparative experiments revealed that O_2 transfer in to the aqueous reaction buffer was overall rate limiting (Figure S5).

Nevertheless, *PeAAOx* performed more than 650.000 catalytic turnovers corresponding to a catalyst loading of less than 0,0002 mol-% or almost $900 \text{ g}_{\text{product}} \text{ g}^{-1} \text{ PeAAOx}$, respectively. The values for catalase are 0,00002 mol-% and $8166 \text{ g}_{\text{product}} \text{ g}^{-1} \text{ Catalase}$, respectively.

Another advantage of the 2LPS approach lies with the facile downstream processing as simple phase separation is sufficient to separate the dodecane-product mixture (in case of full conversion as e.g. shown in Figure 1) from the aqueous reaction buffer. Chromatographic and/or distillative separation of the solvent (dodecane, $b_p = 214^\circ\text{C}$) from the product (*trans*-2-hexenal, $b_p = 145^\circ\text{C}$) is straightforward.

Encouraged by these results, we aimed maximising the catalytic usage of the biocatalyst (i.e. maximising the turnover number). For this, we also decided to avoid any additional organic solvent and use *trans*-2-hexen-1-ol itself as the organic phase (Figure 2). In a first set of experiments, we realised that after approximately 48 h the rate of the oxidation reaction decreased to some extent. Therefore, at intervals, fresh

PeAAOx and catalase were added (in total 6 times throughout the entire experiment) to the aqueous layer summing up to 300 nM and 600 nM of *PeAAOx* and catalase, respectively. This procedure allowed for at least 14 days of stable accumulation of more than 2.6 M of the desired product in the *trans*-2-hexen-1-ol layer. Overall, a superb turnover number of more than 2.2 Million was calculated for *PeAAOx*.

Admittedly, 14 day of reaction time is not practical on the lab-scale or even industrial scale. Also the conversion the starting material of 31% in case of the neat experiment (Figure 2) is not satisfactory as it necessitates further chromatographic separation of the product from the starting material.^[11] Nevertheless, this experiment demonstrates the catalytic potential of *PeAAOx* for the synthesis of *trans*-2-hexenal and possibly further aldehyde products such as benzaldehydes.^[8a,c]

According to the cost estimation by Tufvesson and Woodley^[12] these turnover numbers correspond to a cost contribution of *PeAAOx* of less than 0.1 € mol^{-1} of product (Figure 3, assuming large-scale fermentation of the enzyme). Many other factors will play a role *en route* to economic feasibility of such a process but these numbers again underline the potential of this enzyme for preparative application.

Overall, this contribution demonstrates that oxidase-catalysed alcohol oxidation reactions not only represent a selective approach for the production of aldehydes under non-problematic reaction conditions but also represent an economic promising methodology.

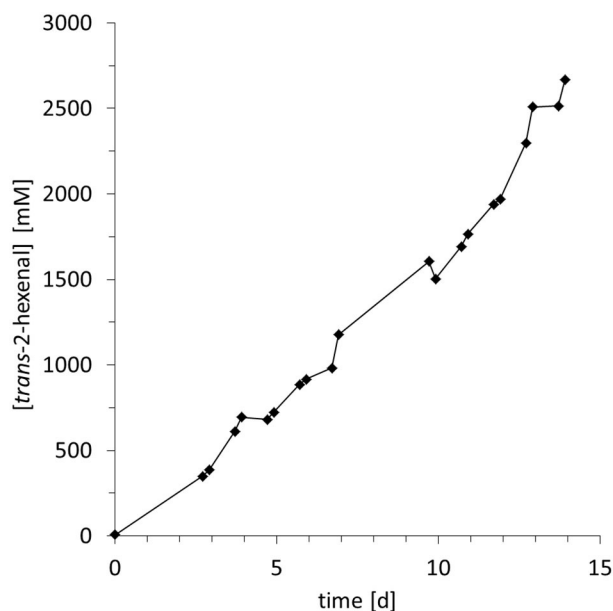


Figure 2. Time course of a long-term oxidation experiment utilising the 2LPS approach. General conditions: $T = 20^\circ\text{C}$, stirring at max speed = 1000 rpm; aqueous phase: 2.5 ml of 50 mM KPi (pH 7), $[\text{PeAAOx}] = 0.3 \text{ }\mu\text{M}_{\text{final}}$ and $[\text{Catalase}] = 0.6 \text{ }\mu\text{M}_{\text{final}}$ (added at 2 d intervals), organic phase: 7.5 ml of [*trans*-2-hexen-1-ol] = 8.4 M (neat), phase ratio: 1:4 (v/v).

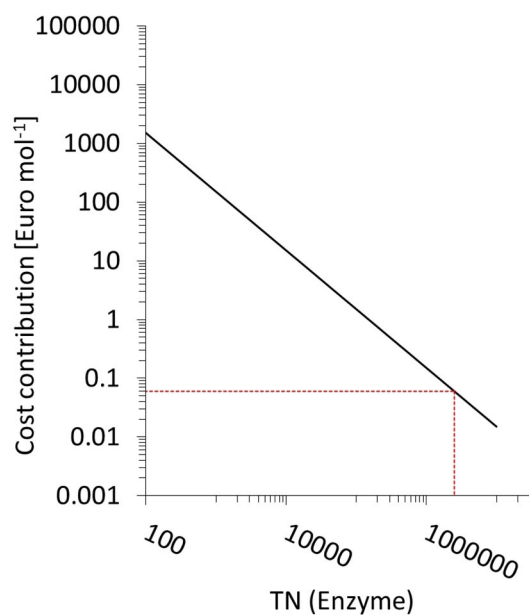


Figure 3. Estimation of the cost contribution of *PeAAOx* to the final product.

Experimental Section

A detailed description of the biocatalyst preparation and -purification as well as a complete description of the experimental and analytical procedures can be found in the Supporting information.

Preparation of the Biocatalyst

PeAAOx was produced by recombinant expression in *Escherichia coli* following a previously established protocol.^[8a] In short: recombinant cells of *E. coli* W3110 pFLAG1-AAO were grown in LB medium at 37 °C. Expression of the enzyme was induced by the addition of IPTG at an OD₆₀₀ of 0.8 followed by an additional incubation for 4 h. PeAAOx was obtained in active form from the insoluble fraction (inclusion bodies) of the cell extract by a refolding and chromatographic purification procedure (please refer to the SI for a full experimental description).

General Conditions for the Biphasic Reaction System

The biphasic reaction systems (1 ml) were composed of an organic solvent layer and an aqueous layer in a 1:1 phase ratio. The organic layer solvent (dodecane, isooctane, ethyl acetate or toluene) was supplemented with 0.5 M *trans*-2-hexen-1-ol. In addition, *trans*-2-hexen-1-ol was used as organic layer. The aqueous phase contained 0.75 μM PeAAOx and 720 U ml⁻¹ (0.1 μM) catalase.

Scale-up and Long-term Experiment in the Biphasic Reaction System

In a scale-up experiment (10 ml), dodecane containing 500 mM *trans*-2-hexen-1-ol and potassium phosphate buffer (50 mM, pH 7.0) containing 0.75 μM PeAAOx and 720 U ml⁻¹ catalase were used in a 1:1 phase ratio. Long-term experiments were performed in glass flasks under magnetic stirring (1,100 rpm). In a total volume of 10 ml, a phase ratio of 1:4 using pure *trans*-2-hexen-1-ol as organic layer and phosphate buffer (50 mM, pH 7) containing 0.05 μM PeAAOx and 720 U ml⁻¹ as aqueous layer was applied. Every two days, 0.05 μM PeAAOx and 720 U ml⁻¹ was added to the solution in the total amount of 0.3 and 0.6 μM of PeAAOx and catalase, respectively

Product Characterisation (*trans*-2-hexenal)

¹H NMR (400 MHz, Chloroform-d) δ 9.51 (d, J = 7.9 Hz, 1H), 6.85 (dt, J = 15.6, 6.8 Hz, 1H), 6.12 (dd, J = 15.6, 7.9 Hz, 1H), 2.36–2.28 (m, 2H), 1.58–1.51 (m, 2H), 0.97 (t, J = 7.4 Hz, 3H). ¹³C NMR (101 MHz, Chloroform-d) δ 194.69, 159.30, 133.56, 35.15, 21.57, 14.10. GC-MS: m/z (99) calc. for C₆H₁₀O [M + H]⁺

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