This article can be cited before page numbers have been issued, to do this please use: M. Romero-Fernandez and F. Paradisi, Green Chem., 2021, DOI: 10.1039/D1GC01095F.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Biocatalytic access to betazole by one-pot multienzymatic system in continuous flow

Maria Romero-Fernandez and Francesca Paradisi

As an alternative to classical synthetic approaches for the production of betazole drug, a one-pot biocatalytic system to this pharmaceutical molecule from its alcohol precursor has been developed. An ω-transaminase, an alcohol dehydrogenase and a water-forming NADH oxidase for in-situ cofactor recycling have been combined to catalyse this reaction yielding 75% molar conversion in batch reaction with soluble enzymes. This multienzyme system was then co-immobilised through a newly established protocol for sequential functionalization of a methacrylate-based porous carrier to enable tailored immobilisation chemistries for each enzyme. This pluri-catalytic system has been set up in a continuous flow packed-bed reactor, yielding a space-time yield up to 2.59 g L⁻¹ h⁻¹ with 15 min residence with constant supply of oxygen for in-situ cofactor recycling through a segmented air-liquid flow. The addition of an in-line catch-and-release column afforded >80% product recovery.

Introduction

The use of isolated enzymes as catalysts has been proposed in the fabrication of important pharmaceutical intermediates. In fact, the ACS Green Chemistry Institute’s Pharmaceutical Round Table has published a list of 10 key research areas aiming to encourage the integration of green chemistry and engineering into the pharmaceutical industry, and biocatalysis can provide methodologies for many of these research areas. Moreover, the use of one-pot biocatalytic cascade systems in synthetic applications displays several economic and environmental benefits: fewer unit operations, minimized solvent and reactor volume, higher throughputs and space-time yields and reduced waste. In addition, it avoids the separation and purification of intermediates, and can drive equilibria towards product formation, consequently enhancing overall yields. One of the challenges currently faced by biocatalysis in synthetic applications stems from enzymes limited stability and water solubility, which hinders their recovery from the aqueous solution and imposes a major cost-contribution to the process. However, significant mitigation of this problem can be achieved by enzyme immobilisation, as it can improve enzyme stability, and leads to the development of heterogeneous biocatalysts, thus increasing enzyme reusability and simplifying product work-up.

Betazole is an established drug in the pharmaceutical industry and a member of the pyrazole derivatives family. This pharmaceutical molecule is an analogue of histamine and a H2-receptor agonist with the ability of stimulating the secretion of gastric acid. Clinical studies confirmed that betazole stimulation allowed an increase in both serum pepsinogen and gastric acid secretion even in patients with chronic Chagas’ disease submitted to surgical treatment, where reduced gastric acid secretion is relevant since chagasic achalasia surgical treatment alters the esophagogastric junction anatomy.

The first synthesis of substituted pyrazoles was carried out in 1883 by Knorr. For betazole, a two steps synthesis was proposed by Jones, R.G. and evolved from a longer synthetic route in 1949. Hydrazine was first reacted with γ-pyrene in an alcohol solution, affording 3-pyrazoleacetaldehyde hydrazone in practically quantitative yields. A Ni-mediated catalytic hydrogenation of the latter compound led to betazole in 80% yield. Nowadays, several relevant synthetic methods are generally used to access the substituted pyrazoles.

As an alternative to classical synthetic approaches, the design of a biocatalytic synthetic route of betazole has therefore potential in increasing the sustainable production of this drug, but to date it has not been explored. In particular, the biocatalytic synthesis of the primary amine of betazole is relevant, since biocatalysis is a proven powerful tool in the synthesis of (enantiopure) amines, which are key intermediates in the fabrication of numerous active pharmaceutical ingredients (APIs). Both, ω-transaminases (TA) and amine dehydrogenases (AmDHs) catalyse the selective production of primary amines from carbonyl compound. Furthermore, the synthesis of amines from the corresponding alcohols offers significant advantages since the alcohol is mostly easily accessible. In fact, different metal-catalysed methodologies have been proposed to carry out this reaction. No enzyme is however known to directly aminate an alcohol,
and multienzyme systems have been proposed to catalyse this reaction in a two-steps process: alcohol oxidation to form a carbonyl group, followed by reductive amination of the latter to produce a primary amine. Despite the apparent simplicity, the technical challenges to establish a reliable, sustainable and highly yielding system are significant. To date, only very few such multienzyme systems have been reported: galactose oxidase/TA/alanine dehydrogenase (AlaDH),22 alcohol dehydrogenase (ADH)/TA/AlaDH;23,24 ADH/TA/lactate dehydrogenase;24 ADH/AmDH,25 and alcohol oxidase/TA/AlaDH/catalase.26

The ADH-catalysed oxidation of alcohols requires stoichiometric amounts of oxidised nicotinamide adenine dinucleotide cofactors i.e. NAD(P)⁺, which represent a major cost contribution to the system. Therefore, an in-situ regeneration system for the oxidised cofactor is essential for the cost-efficiency of the process. The integration of water-forming NAD(P)H oxidase (NOX) enzyme would appear to be ideal,27 as NAD(P)H are oxidized at the expense of the most innocuous and cheapest oxidant, O₂, by producing H₂O as by-product.27 Several water-forming NOX have been described in literature.28–30 However, the application of water-forming NOX to in-situ cofactor recycling in continuous flow methodologies, which are increasing in popularity in the pharmaceutical industry, is not straightforward. O₂ transfer from the gas to the aqueous liquid phase faces several well-known restrictions.31 Although efficient O₂-liquid transfer can be achieved in conventional small-scale batch reactors by high levels of aeration and agitation,32 providing O₂ to enzymatic reactions is specially challenging in standard biocatalytic flow reactors operating only with liquid phase, where there is little gas (air)-liquid contact.

The aim of this work is to expand the applicability of synthetic biocatalysis overcoming existing challenges which limit its implementation, adopting as a case-study the sustainable production of betazole, a pyrazole derivative of pharmacological interest. For this purpose, biocatalysis and flow chemistry have been integrated to develop a one-pot multienzyme system in a continuous flow packed-bed reactor (PBR). Moreover, a novel protocol of multienzyme co-immobilisation on a polymethacrylate-based porous beads carrier has been developed. This system has been successfully applied to catalyse the direct synthesis of the primary amine of betazole from the corresponding alcohol.

Results and discussion

One-pot multienzymatic cascade combining HLADH, HEWT and LpNOX

A one-pot enzymatic cascade reaction to catalyse the direct synthesis of the primary amine of betazole from the corresponding alcohol was designed: ADH and TA, together with water-forming NOX as an in-situ cofactor regeneration system. The use of the S-selective horse liver alcohol dehydrogenase (HLADH),31,34 the S-selective ω-transaminase from Halomonas elongata (HeWT),35–37 and the water-forming NADH oxidase from Lactobacillus pentosus (LpNOX)29,38 was proposed (Scheme 1).

Initially, the catalytic activity of this multienzyme system on the alcohol group of 2-(1H-pyrazol-3-yl)ethanol was tested in batch reactions at 10 mM scale with a catalytic amount of NAD⁺ (0.1 mol equivalents), using isopropylamine (IPA) as amino-donor (2 mol equivalents) and the required enzymatic cofactors. High molar conversion (m. c.), 75%, to betazole was achieved after 21.5 hours under the specified reaction conditions (Table 1). HLADH and HEWT can therefore catalyse the functional group interconversion of the alcohol of 2-(1H-pyrazol-3-yl)ethanol into the corresponding primary amine to yield betazole in one-pot system. The presence of LpNOX, despite its known low stability,29 increases the reaction velocity, meaning that in-situ NAD⁺ recycling pushes the reaction equilibrium towards alcohol oxidation (Fig. S1). This is likely due to the high redox potential of the O₂/H₂O couple that results in a strong thermodynamic driving force.27

Table 1 Synthesis of betazole drug in batch reactions catalysed by soluble HLADH (2 mg ml⁻¹), HEWT (0.2 mg ml⁻¹), and LpNOX (0.1 mg ml⁻¹). Reaction conditions: 10 mM 2-(1H-pyrazol-3-yl)ethanol in phosphate buffer (0.05 M, pH 8), 1 mM NAD⁺, 20 mM IPA, 0.1 mM flavin adenine dinucleotide (FAD), 0.1 mM pyridoxal 5’-phosphate (PLP). T = 30°C. Reaction volume = 5 mL.

<table>
<thead>
<tr>
<th>Substrate concentration (mM)</th>
<th>1 h</th>
<th>5 h</th>
<th>21.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>50</td>
<td>66</td>
<td>75</td>
</tr>
</tbody>
</table>

© Determined by HPLC

Tailored multienzymatic co-immobilised system

To increase the reusability of this multienzyme system in continuous flow and simplify product work-up,39 the immobilisation of HLADH, HEWT and LpNOX was achieved. LpNOX, in particular, proved to be highly challenging. Among all the immobilisation methods proposed, covalent immobilisation offers significant advantages as it can improve enzyme stability by rigidification throughout their structure,30 and it was successfully attained for HLADH44,41 and HEWT.13 However, despite extensive testing with both epoxy and glyoxyl chemistries, the obtained recovered activity of LpNOX was very low even at low catalyst loading (5mg/g) achieving a maximum of 23% with partial immobilisation yield (Table S1). An alternative immobilisation method for LpNOX based on ionic
adsorption was selected, and this increased the recovered activity to 33% while providing the added benefit of enabling the desorption of the catalyst for replacement with fresh enzyme following exhaustion of the activity.

In addition to the high conversions obtained by multistep flow biocatalysis in serial reactors for the synthesis of different molecules,37 one-pot flow biocatalysis based on a multistep enzymatic synthesis in a single reactor offers significant advantages.1,4,20 Moreover, immobilising the different enzymes on the same intrapore bead surface improves further the performance of biocatalytic cascade systems due to facilitated intrapore diffusion of substrates and cofactors between different enzyme active sites.42–44 Although co-immobilisation of multienzyme systems is highly challenging since there is no universal immobilisation chemistry or carrier, selective and optimal immobilisation of the different enzymes can be achieved by functionalization of the carrier surface with different reactive groups.42,44,45 However, no examples have been reported on polymethacrylate-based carriers, which are relevant for continuous flow applications.36,46,47 Therefore, a strategy for the co-immobilisation of this multienzyme system was developed on a porous beads carrier based on a polymethacrylate polymer matrix. HLADH, HEWT and LpNOX enzymes were co-immobilised using tailored immobilisation chemistries for each of them. This was achieved by a novel protocol of sequential functionalization of a methacrylate carrier with different reactive groups (Scheme 2) to pursue optimal immobilisation for each of the enzymes and facilitate the diffusion of substrates between them. The epoxy groups displayed on the carrier surface were partially hydrolysed to diol groups in a controlled manner, and diol groups were then oxidised to glyoxyl groups. The carrier initial epoxy group density was determined to be 94 µmol g\textsuperscript{-1} carrier\textsuperscript{-1}. After partial hydrolysis with H\textsubscript{2}SO\textsubscript{4} and oxidation with NaIO\textsubscript{4} treatment, the resulting glyoxyl group density was 78.3 µmol g\textsuperscript{-1} carrier\textsuperscript{-1}, of which 14% resulted from the transformation of epoxy into glyoxyl groups, and 86% resulted from the transformation of diol groups initially present in the carrier into glyoxyl groups. The initial loading of epoxy plus diol groups of the carrier was determined to be 161 µmol g\textsuperscript{-1} (Table S2). Since this strategy also makes use of the diol groups already present in the carrier, it offers the advantage of a better usage of the carrier surface and a higher efficiency of the resulting catalyst.

The resulting glyoxyl groups were used to immobilise the first enzyme, HLADH, by the multipoint-covalent attachment methodology previously described (Fig. S2).30,46 This methodology is developed under alkaline conditions, where amine groups from lysine residues of the enzyme react with glyoxyl groups to form a Schiff’s base which yields a secondary amine upon reduction with NaBH\textsubscript{4}.40 A minor proportion of HLADH was expected to immobilise via epoxy groups (Fig. S4). Some of the remaining epoxy groups (~12%) were then modified to amine groups upon reaction with ethylenediamine (EDA). The second enzyme, HEWT, was covalently immobilised by the two-steps amino-epoxy strategy previously reported.48,49 This two-steps approach is based on a first stabilisation of enzyme molecules by ionic interaction between their anionic residues and amine groups of the carrier, prior to the covalent bond formation between their nucleophilic groups and nearby epoxy groups on the carrier.48 The aim of this strategy is to overcome the low reactivity of epoxy groups found in enzyme immobilisation.48 Upon addition of polyethyleneimine (PEI), at
least some of the unreacted epoxyl groups covalently reacted with the amine groups of PEI, which was also used as a scaffold for the immobilisation by ionic adsorption of the third enzyme, LpNOX. With this strategy, ionic adsorption of enzyme molecules is established by ionic interactions between amine groups of PEI and aspartic and glutamic acid residues of the enzyme. Overall good values of immobilisation yield and recovered activity were obtained for the three enzymes (Table 2). Note that the low reported recovered activity for the HLADH could be possibly attributed to a mass transfer limitation at this high catalyst loading (Fig. S3). High stability of HLADH and HEWT was achieved upon covalent immobilisation, and sufficient stability of LpNOX was reached by ionic adsorption on PEI (Fig. S5). As a proof of concept, LpNOX could be efficiently desorbed by incubation with 0.2 M NaCl and fresh LpNOX could be immobilised on the same catalyst yielding 35% of the initial specific immobilised activity while preserving HLADH and HEWT immobilised activities (Table S3) demonstrating the feasibility of the approach.

Table 2 Immobilisation of 20 mg HLADH, 5 mg HEWT and 5 mg LpNOX per gram of functionalized porous beads carrier based on a polymethylacrylate matrix.

<table>
<thead>
<tr>
<th>Immobilisation yield (%)</th>
<th>Recovered activity (%)</th>
<th>Specific immobilised activity (U g carrier⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLADH</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>HEWT</td>
<td>93</td>
<td>34</td>
</tr>
<tr>
<td>LpNOX</td>
<td>85</td>
<td>33</td>
</tr>
</tbody>
</table>

* Immobilisation yield (%), recovered activity (%) and specific immobilised activity (U g carrier⁻¹) were calculated as described in Experimental section.

The catalytic activity of the resulting co-immobilised multienzyme system was evaluated on the alcohol group of 2-(1H-pyrazol-3-yl)ethanol in batch reactions under the same conditions as used with soluble enzymes. High m. c., 70%, to the corresponding primary amine was also achieved after 3 hours of reaction under specified conditions (Table 3). To demonstrate the reusability of this co-immobilised HLADH-HEWT-LpNOX biocatalyst, it was used to catalyse 3 batch reaction cycles. Remarkably, the catalytic efficiency of this multienzyme system was practically unaltered for 2 batch reaction cycles of 3 h (Table 3). After the second reaction cycle, the catalytic efficiency of the co-immobilised multienzyme system decreased considerably. This is likely due to the low stability of LpNOX as the activity of immobilised LpNOX on this multienzyme system dropped to 35% after 2 h of incubation at bath reaction conditions of temperature, pH and agitation, and to 1.6% after 24 h (Fig. S5).

Table 3 Synthesis of betazole drug in batch reactions cycles catalysed by 0.2 g of the (re-used) co-immobilised multienzyme system consisting of HLADH, HEWT and LpNOX per mL of reaction. Reaction conditions: 10 mM 2-(1H-pyrazol-3-yl)ethanol in phosphate buffer (0.05 M, pH 8), 1 mM NAD⁺, 20 mM IPA, 1 mM FAD, 0.1 mM PLP. T = 30 °C. Reaction volume = 1 mL.

<table>
<thead>
<tr>
<th>Batch reaction cycle</th>
<th>m. c. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td>63</td>
</tr>
<tr>
<td>3 h</td>
<td>70</td>
</tr>
<tr>
<td>5 h</td>
<td>71</td>
</tr>
</tbody>
</table>

Flow biocatalysis with external oxygen supply

A continuous flow PBR was set-up using this co-immobilised multienzyme system and optimized for the functional group interconversion of the alcohol of 2-(1H-pyrazol-3-yl)ethanol into the corresponding primary amine to synthesise betazole drug. A liquid phase containing the substrate at 10 mM, catalytic amount of NAD⁺ (0.1 mol equivalents), IPA (2 mol equivalents), and the required enzymatic cofactors, was flowed into the packed-bed reactor with a residence time of 30 min, obtaining 10% m. c. (Scheme 3, black).

In systems requiring O₂ as a substrate, as is the case for NOX catalysts, standard flow reactors can be highly limiting unless constant supply of O₂ to the liquid phase can be guaranteed (e.g. tube-in-tube reactors, segmented air-liquid flow reactors), or a strategy to increase the O₂ concentration in the liquid phase (e.g. pressurized flow reactors) is implemented. A simple solution when a packed-bed reactor is employed can be achieved with segmented gas–liquid flow strategy composed of substrate solution/air. To date this was only reported with whole cells catalysts. Here this approach was trialled with cell-free immobilised catalysts to ensure a constant reservoir of oxygen to supply the liquid phase for the LpNOX-catalysed in-situ cofactor regeneration in this PBR (Scheme 3, red).

A segmented 50:50 substrate solution/air flow was flowed into the PBR with 15 min residence time. This biphasic flow allowed alcohol amination with 50% m. c. To our knowledge, only one example of enzymatic amination of alcohols in continuous flow has been previously reported. However, the present work is the first example in literature of a multienzyme system applied to amination of alcohols in continuous flow that uses TA, which is mostly relevant given the broad range of applications of this type of enzymes in the production of APIs and their intermediates.
Recirculation of the reaction mixture through the PBR, increased the m. c. to 72% highlighting a possible limitation on the residence time. A recirculation strategy of the reaction mixture (Scheme 3, green) was then further implemented.

A biphasic flow of 50:50 substrate solution/air and a residence time of 4x15 min pushed the conversion to 84%. Very low increment of space time yield (STY) with further passes was observed after the first pass, where substrate concentration drops to 5 mM. This suggests a K_H issue and poor enzymatic efficiency below such threshold (<5 mM) (Fig. S6). When the reaction scale was increased to 50 mM, and the same flow reaction conditions were applied, 23% m. c. to betazole was obtained after 1 pass through this PBR and 66% after 4 passes. STY and catalyst productivity were higher at 50 mM scale, although conversion was lower (Table 4). At 30 mM scale, a compromise between conversion and space time yield was found. At this substrate concentration, 16% m. c. to betazole was obtained after 1 pass and 69% after 4 passes. After 20 passes, 85% m.c. was achieved.

Table 4 Continuous biocatalytic production of betazole drug implementing segmented liquid-gas flow composed of substrate solution/air and recirculation. Recirculated solution volume 9.151 mL. Reactor volume 3.66 mL. Residence time 15 min.

<table>
<thead>
<tr>
<th>Scale (mM)</th>
<th>Residence time (min)</th>
<th>M. c. (%)</th>
<th>STY (g betazole L^-1 h^-1)</th>
<th>Catalyst productivity (µmol betazole h^-1 mg enzyme^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1 x 15 min</td>
<td>50</td>
<td>1.11</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>2 x 15 min</td>
<td>72</td>
<td>1.61</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>4 x 15 min</td>
<td>84</td>
<td>1.86</td>
<td>0.67</td>
</tr>
<tr>
<td>30</td>
<td>1 x 15 min</td>
<td>16</td>
<td>1.07</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>2 x 15 min</td>
<td>35</td>
<td>2.28</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>4 x 15 min</td>
<td>69</td>
<td>4.53</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>20 x 15 min</td>
<td>85</td>
<td>5.60</td>
<td>2.07</td>
</tr>
<tr>
<td>50</td>
<td>1 x 15 min</td>
<td>23</td>
<td>2.59</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>2 x 15 min</td>
<td>44</td>
<td>4.91</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>4 x 15 min</td>
<td>66</td>
<td>7.37</td>
<td>2.72</td>
</tr>
</tbody>
</table>

* Determined by HPLC. † STY (g betazole L^-1 h^-1) and Catalyst productivity (µmol betazole h^-1 mg enzyme^-1) calculated as described in ESI.

Assuming a volumetric composition of oxygen in air of 21%, the input oxygen flow rate was 0.025 mL min^-1. At 30ºC and 1 atm, this would be equivalent to 1.012 µmol oxygen min^-1 flowing to the PBR in the gas phase. The molar conversion to betazole at 10 mM scale and 15 min residence time after 1 pass was 50%, which corresponded to 0.60 µmol product min^-1. At this reaction scale, the input NAD^+ concentration was 1 mM, therefore, 0.12 µmol NAD^+ min^-1 was flowed into the system. Since the stoichiometry of the ADH-catalysed alcohol oxidation and concomitant NAD^+ reduction is 1 to 1, 0.48 µmol NAD^+ min^-1 must be recycled in 1 pass (at least). This would indicate that 0.24 µmol oxygen min^-1 were consumed in the enzymatic reaction under these conditions. In case of the 50 mM scale reactions, the oxygen intake rate by the enzymatic reaction in the first pass was estimated to be 0.39 µmol oxygen min^-1. Moreover, a segmented liquid-gas flow was required to ensure a constant reservoir of oxygen to supply the liquid phase and make the reaction work (only 10% m. c. was obtained without air supply in the 10 mM reaction). Therefore, 0.24 µmol oxygen min^-1 were then supplied from the gas to the liquid phase in the 10 mM scale reaction, and 0.39 µmol oxygen min^-1 in the 50 mM scale reaction, under the applied flow conditions, and consumed by the enzymatic reaction.

At 50 mM scale without any recirculation, this one-pot multienzymatic system yields 2.59 g betazole L^-1 h^-1 in continuous flow, which is mostly relevant since it represents the first example of a biocatalytic synthetic step in the fabrication of betazole. As opposed to classical synthetic routes to this pharmaceutical molecule, this metal-free biocatalytic approach allows the synthesis of the primary amine of betazole from the corresponding alcohol by means of a highly yielding system using only water as solvent at 30ºC and atmospheric pressure, and without the formation of unwanted side-products. Moreover, the co-immobilisation strategy of this multienzyme system increased biocatalyst reusability, thus increasing the catalyst productivity in continuous flow. Considering a window of 5 h of operational stability of the co-immobilised multienzyme system under specified flow conditions (Table 4), the accumulated catalyst productivity is 4.8 µmol betazole mg enzyme^-1.

Finally, a step to trap the primary amine-containing molecules (which include also unreacted IPA as well as betazole) was integrated in the system. A tailor-made aldehyde resin was packed in a column and placed downstream of the recirculating flow reaction. This amine catch-and-release step had an efficiency of 93% with respect to the recovered betazole after desorption with 0.2% HCl solution (Fig. S8). The IPA could be successfully removed by freeze-drying the eluted fractions, as shown by 1H NMR and 31P NMR. The betazole-HCl salt form of the product was isolated with an apparent 94% yield. The difference with the reaction conversion is due to the presence of residual phosphate salts from the buffer as confirmed by 31P NMR.

Conclusions

This work represents the first example of implementation of a biocatalysed synthetic step in the production of betazole drug, and one of the few examples of biocatalytic amination of alcohols in a continuous flow PBR, and the first one using TA for reductive amination of the formed carbonyl group, which is mostly relevant given the broad range of applications of TA in the production of APIs and their intermediates. This biocatalytic strategy could also be integrated with other bio/chemo-catalysed steps to design alternative routes of synthesis of this drug molecule. The developed segmented gas-liquid flow strategy ensured a constant reservoir of oxygen which further promotes the application of water-forming NOX as recycling systems in continuous flow PBRs, thus further contributing to the combination of biocatalysis and flow technologies. The immobilisation of three different enzymes with...
tailored chemistries shows how optimal conditions can be achieved on the same carrier even for catalysts with very different stability profiles. While the stability of LpNOX was not significantly improved, the possibility of replenishing the exhausted catalyst, without affecting the other enzymes, was demonstrated. The method described here has therefore substantial potential in the synthesis of pure primary amines from the corresponding alcohol intermediates within the production of numerous APIs, and in the integration of greener synthetic approaches in the pharmaceutical industry.

**Experimental section**

**Materials**

All chemicals and reagents used for this work were purchased from Sigma-Aldrich unless specified otherwise. GoTaq® DNA polymerase was purchased from Promega. Nhel-HF®, EcoRV-HF® and T4 DNA ligase were acquired from New England Biolabs. QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit were purchased from Qiagen. XL10-Gold® Ultracompent cells were purchased from Agilent. E. coli BL21(DE3) Star competent cells were purchased from Thermo Fisher Scientific. Anhydroractracrylase was purchased from Cyman Chemical Company. StrepTrap HP 5 mL and HisTrap HP 1 mL columns were acquired from GE Healthcare. Nicotinamide adenine dinucleotide oxidised (NAD⁺) and reduced (NADH) forms were obtained from Apollo Scientific. Relisorb® EP400SS was purchased from Resindion. Polyethyleneimine (PEI) (M.N. 60000, 50% aq. Solution, branched) was purchased from Acros Organics. 2-(1H-pyrazol-3-yl)ethanol and betazole were acquired from Fluorochem.

**Cloning of HLADH**

The gene coding the horse alcohol dehydrogenase-E-isoenzyme (GenBank accession number M64864) was synthesized by GeneArt. Gene Synthesis service from Thermo Fisher Scientific. The gene was amplified by PCR with GoTaq® DNA polymerase with the oligonucleotide primers FWD-HLADH (5' - AATGGCTAGTGAGCCACCGACTTCGAAAAAGGGCGCATGAGCACGCGAC-3') and RV-HLADH (5' - TAGTTAGATATCATAAAAAGTGCAAG-3'). The primers were designed to incorporate Nhel and EcoRV restriction sites, respectively (underlined). The manufacturer’s protocol for GoTaq® DNA polymerase PCR in 50 µL reaction was used. PCR was carried out with 10 µL 5x GoTaq® buffer; 2.5 µL 10 mM FWD-HLADH primer; 2.5 µL 10 mM 10 mM RV-HLADH primer; 32.5 µL nuclease-free water; 1 µL 100 ng µL⁻¹ plasmid containing the HLADH gene; and 1 µL GoTaq® DNA polymerase. PCR conditions were 2 min of at 72°C and 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C and 2 min extension 72°C. The 35 cycles were followed by 7 min of final elongation at 72°C. The amplified DNA was purified with QiAquick PCR Purification Kit. The purified PCR product was digested with Nhel-HF® and EcoRV-HF® for 2 h at 37°C. The digested gene was gel-extracted using QiAquick Gel Extraction Kit and cloned into the pASK-IBA5plus vector harbouring Lactobacillus brevis ADH, kindly donated by Prof. Kroutil, digested with the same restriction enzymes. Ligation was developed with T4 DNA ligase following the manufacturer’s guidelines and the resulting product was used to transform E. coli XL10-Gold® Ultracompent cells. The construct was verified by sequencing and the obtained plasmid was named pASK-IBA5plus-HLADH.

**Enzyme expression**

The plasmid harbouring HLADH (pASK-IBA5plus-HLADH) was transformed into E. coli BL21(DE3) Star. 1 L flasks containing 300 mL of Terrific Broth media were inoculated with 10 mL of an overnight culture in LB and left growing at 37°C and 180 rpm until OD₆₀₀ reached 0.6–0.7. At that point, the expression was induced with 0.4 µM anhydrotetracycline (final concentration) and the cultures were left to grow for 16 h at 20°C and 180 rpm. The previously reported expression protocol of HEWT was followed. For LpNOX, a single colony of E. coli BL21 (DE3) Star cells previously transformed with the plasmid pET28a-LpNOX, kindly donated from Prof. Sieber, was inoculated in autoinduction media ZYP-5052. The cultures were left to grow at 37°C and 180 rpm for 20 h. The cells were collected by centrifugation at 4500 rpm and stored at -20°C until further used.

**Enzyme purification**

The cells containing expressed HLADH enzyme were resuspended in 20 mM sodium phosphate buffer, 0.28 M NaCl, 6 mM KCl pH 7.4. Cells were disrupted by sonication with pulses of 5 seconds on and 5 seconds off at 60% amplitude for 12 min. The insoluble fraction was separated by centrifugation at 14500 rpm for 60 min. The supernatant was filtered (0.45 µm) and loaded into a StrepTrap HP 5 mL column using ÄKTA™ start FPLC (GE Healthcare). The protein was eluted with 20 mM sodium phosphate buffer, 0.28 M NaCl, 6 mM KCl and 2.5 mM d-desthiobiotin pH 7.4. HLADH was dialysed twice against 50 mM Tris buffer pH 8 at 4°C. For HEWT, cells were resuspended in 50 mM potassium phosphate buffer, 0.1 M NaCl, 0.1 mM PLP and 30 mM imidazole pH 8. The same sonication and centrifugation conditions as in case of HLADH were used. The supernatant was filtered (0.45 µm) and HEWT was purified using ÄKTA™ start FPLC as previously described. HEWT was dialysed twice against 50 mM potassium phosphate buffer 0.1 mM PLP pH 8 at 4°C. Cells containing expressed LpNOX enzyme were resuspended in 50 mM potassium phosphate buffer, 0.5 M NaCl, 20 mM imidazole, 10% (v/v) glycerol, 0.1 mM flavin adenine dinucleotide (FAD) pH 8. After cell disruption and insoluble fraction separation under the same conditions as before, the filtered supernatant was loaded into a HisTrap HP 1 mL column using ÄKTA™ start FPLC. The protein was eluted with 50 mM potassium phosphate buffer, 0.5 M NaCl, 0.5 M imidazole, 10% (v/v) glycerol, 0.1 mM FAD pH 8, and dialysed twice against 50 mM Tris buffer 0.01 mM FAD pH 7.5 at 4°C.

**Enzymatic activity assays and determination of protein concentration**

All enzyme assays were performed in triplicate in 96-well microplates (unless specified otherwise) using Epoch 2 Microplate Spectrophotometer (Biotek). For HLADH and HEWT, the activity assays were performed as previously indicated. The plasmid IBA5plus vector harbouring Lactobacillus brevis ADH, kindly donated by Prof. Kroutil, digested with the same restriction enzymes. Ligation was developed with T4 DNA ligase following the manufacturer’s guidelines and the resulting product was used to transform E. coli XL10-Gold® Ultracompent cells. The construct was verified by sequencing and the obtained plasmid was named pASK-IBA5plus-HLADH.

**Enzyme expression**

The plasmid harbouring HLADH (pASK-IBA5plus-HLADH) was transformed into E. coli BL21(DE3) Star. 1 L flasks containing 300 mL of Terrific Broth media were inoculated with 10 mL of an overnight culture in LB and left growing at 37°C and 180 rpm until OD₆₀₀ reached 0.6–0.7. At that point, the expression was induced with 0.4 µM anhydrotetracycline (final concentration) and the cultures were left to grow for 16 h at 20°C and 180 rpm. The previously reported expression protocol of HEWT was followed. For LpNOX, a single colony of E. coli BL21 (DE3) Star cells previously transformed with the plasmid pET28a-LpNOX, kindly donated from Prof. Sieber, was inoculated in autoinduction media ZYP-5052. The cultures were left to grow at 37°C and 180 rpm for 20 h. The cells were collected by centrifugation at 4500 rpm and stored at -20°C until further used.

**Enzyme purification**

The cells containing expressed HLADH enzyme were resuspended in 20 mM sodium phosphate buffer, 0.28 M NaCl, 6 mM KCl pH 7.4. Cells were disrupted by sonication with pulses of 5 seconds on and 5 seconds off at 60% amplitude for 12 min. The insoluble fraction was separated by centrifugation at 14500 rpm for 60 min. The supernatant was filtered (0.45 µm) and loaded into a StrepTrap HP 5 mL column using ÄKTA™ start FPLC (GE Healthcare). The protein was eluted with 20 mM sodium phosphate buffer, 0.28 M NaCl, 6 mM KCl and 2.5 mM d-desthiobiotin pH 7.4. HLADH was dialysed twice against 50 mM Tris buffer pH 8 at 4°C. For HEWT, cells were resuspended in 50 mM potassium phosphate buffer, 0.1 M NaCl, 0.1 mM PLP and 30 mM imidazole pH 8. The same sonication and centrifugation conditions as in case of HLADH were used. The supernatant was filtered (0.45 µm) and HEWT was purified using ÄKTA™ start FPLC as previously described. HEWT was dialysed twice against 50 mM potassium phosphate buffer 0.1 mM PLP pH 8 at 4°C. Cells containing expressed LpNOX enzyme were resuspended in 50 mM potassium phosphate buffer, 0.5 M NaCl, 20 mM imidazole, 10% (v/v) glycerol, 0.1 mM flavin adenine dinucleotide (FAD) pH 8. After cell disruption and insoluble fraction separation under the same conditions as before, the filtered supernatant was loaded into a HisTrap HP 1 mL column using ÄKTA™ start FPLC. The protein was eluted with 50 mM potassium phosphate buffer, 0.5 M NaCl, 0.5 M imidazole, 10% (v/v) glycerol, 0.1 mM FAD pH 8, and dialysed twice against 50 mM Tris buffer 0.01 mM FAD pH 7.5 at 4°C.

**Enzymatic activity assays and determination of protein concentration**

All enzyme assays were performed in triplicate in 96-well microplates (unless specified otherwise) using Epoch 2 Microplate Spectrophotometer (Biotek). For HLADH and HEWT, the activity assays were performed as previously indicated. The plasmid IBA5plus vector harbouring Lactobacillus brevis ADH, kindly donated by Prof. Kroutil, digested with the same restriction enzymes. Ligation was developed with T4 DNA ligase following the manufacturer’s guidelines and the resulting product was used to transform E. coli XL10-Gold® Ultracompent cells. The construct was verified by sequencing and the obtained plasmid was named pASK-IBA5plus-HLADH.
activity was defined as the oxidation of 1 μmol of cofactor per minute. For the immobilised enzyme, the activity was measured using the same conditions as for the free enzyme. For HLADH and HEWT, 20 mg of the immobilised biocatalyst were added to a 10 mL reaction mixture. At regular intervals of time during 10 min, a sample was taken to measure the absorbance at 340 nm or 245 nm, for HLADH or HEWT, respectively. For LpNOX, 20 mg of the immobilised biocatalyst were added to 40 mL of reaction mixture, and the same protocol was followed. The specific activity of HLADH, HEWT and LpNOX immobilised biocatalysts (U g\textsuperscript{-1}) was defined as the formation of NADH in μmol per minute and g of biocatalyst (HLADH); the formation of acetonophenone in μmol per minute and g of biocatalyst (HEWT); and the depletion of NADH in μmol per minute and g of biocatalyst (LpNOX). HLADH and HEWT protein concentration were determined by UV absorption at 280 nm using the Epoch Take3 Micro-Volume Plate. The extinction coefficients 22460 M\textsuperscript{-1} cm\textsuperscript{-1} and 62340 M\textsuperscript{-1} cm\textsuperscript{-1}, at 280 nm, measured in water, were respectively estimated for HLADH and HEWT by ExPaSy ProtParam tool, accessible from the ExPaSy website (www.expasy.ch). The LpNOX protein concentration was determined using a Bradford assay with bovine serum albumin (BSA) as standard, as previously reported.  

Sequential functionalization of methacrylate carrier and co-immobilisation of HLADH, HEWT and LpNOX  
3 g of Relisorb® EP400SS were resuspended in 30 mL of 0.1 M H\textsubscript{2}SO\textsubscript{4}, and the suspension was incubated for 30 min at room temperature under mild agitation. The hydrolysed carrier was filtered, washed with H\textsubscript{2}O and resuspended in 30 mL of 10 mM NaO4. After 2 h incubation at room temperature under mild agitation, the resulting carrier, was filtered and washed with H\textsubscript{2}O (Scheme 2, A). Resulting glyoxyl groups were quantified as previously described.  
A 30 mL solution containing 60 mg of pure HLADH in 100 mM NaHCO\textsubscript{3} pH 10 was added to the resin containing glyoxyl groups. The suspension was incubated for 1 h at 4º C under mild agitation, and 30 mg of NaBH\textsubscript{4} were then added and further incubated for 30 min at 4º C under mild agitation. HLADH activity in the remaining liquid phase before NaBH\textsubscript{4} addition was measured. The immobilised HLADH biocatalyst was filtered and washed with H\textsubscript{2}O (Scheme 2, B). 2 samples of 20 mg of the immobilised HLADH biocatalyst were taken to determine the specific activity of the immobilised HLADH enzyme (in duplicate) as described before. The rest was added to a 30 mL solution of 300 mM ethylenediamine (EDA) in 100 mM NaHCO\textsubscript{3} pH 8.5. The suspension was incubated for 2 h at room temperature under mild agitation. The EDA-modified immobilised HLADH biocatalyst was filtered and washed with H\textsubscript{2}O (Scheme 2, C). A 30 mL solution containing 15 mg of pure HEWT in 50 mM potassium phosphate buffer 0.1 mM pyridoxal 5’-phosphate (PLP) pH 8 was added to this biocatalyst. After 4 h incubation at 4º C under mild agitation, the resulting co-immobilised HEWT-HLADH biocatalyst was filtered, washed with H\textsubscript{2}O (Scheme 2, D), and added to a 60 mL solution of 0.05 g PEI mL\textsuperscript{-1} in 25 mM potassium phosphate buffer pH 7.5. The suspension was incubated for 16 h at room temperature under mild agitation, and the resulting co-immobilised HEWT-HLADH biocatalyst was filtered and washed with H\textsubscript{2}O (Scheme 2, E). 4 samples of 20 mg of the co-immobilised HEWT-HLADH biocatalyst were taken to determine the specific activity of the immobilised HEWT and HLADH enzymes (in duplicate). A 30 mL solution containing 15 mg of pure LpNOX in 25 mM potassium phosphate buffer 0.1 mM FAD pH 7 was added to this biocatalyst. After 4 h incubation at 4º C under mild agitation, the resulting co-immobilised LpNOX-HEWT-HLADH biocatalyst was filtered and washed with H\textsubscript{2}O (Scheme 2, F). 2 samples of 20 mg of the co-immobilised LpNOX-HEWT-HLADH biocatalyst were taken to determine the specific activity of the immobilised LpNOX enzyme (in duplicate). The immobilised activity (U g\textsuperscript{-1}) was calculated as the difference between initial offered activity per gram of carrier and remaining activity in the liquid phase per gram of carrier at immobilisation end point. The immobilisation yield (%) was calculated as the ratio between immobilised activity and initial offered activity per gram of carrier. The recovered activity (%) was calculated as the ratio between the specific activity of the immobilised enzyme and the immobilised activity.  

Batch reaction of biocatalytic synthesis of betazole drug from 2-(1H-pyrazol-3-yl)ethanol  
Batch reactions with pure soluble enzymes were performed at 30º C in 5 mL of reaction mixture containing 10 mM 2-(1H-pyrazol-3-yl)ethanol, 1 mM NAD\textsuperscript{+}, 200 mM IPA, 0.1 mM PLP, 0.1 mM FAD, 50 mM potassium phosphate buffer pH 8, HLADH (2 mg mL\textsuperscript{-1}), HEWT (0.2 mg mL\textsuperscript{-1}), and LpNOX (0.1 mg mL\textsuperscript{-1}). Batch reactions with the co-immobilised multi-enzyme system were performed at 30º C in 1 mL of reaction mixture containing 10 mM 2-(1H-pyrazol-3-yl)ethanol, 1 mM NAD\textsuperscript{+}, 200 mM IPA, 0.1 mM PLP, 0.1 mM FAD, 50 mM potassium phosphate buffer pH 8, and 0.2 g of the co-immobilised LpNOX-HEWT-HLADH biocatalyst. The reusability test of the co-immobilised LpNOX-HEWT-HLADH biocatalyst was developed in 3 h reaction cycles under the same conditions. After a reaction cycle, the co-immobilised LpNOX-HEWT-HLADH biocatalyst was filtered, washed with H\textsubscript{2}O and added to fresh reaction mixture. The reactions were monitored by HPLC, as described in Analytical methods section.  

Continuous flow reaction of biocatalytic synthesis of betazole drug from 2-(1H-pyrazol-3-yl)ethanol  
An Omnifit glass column (6.6 mm i.d. x 100 mm length) was filled with 3 g the co-immobilised LpNOX-HEWT-HLADH biocatalyst to set-up a PBR (reactor volume, 3.66 mL). In all the reactions performed, temperature was set to 30º C. A 10 mM substrate solution (2-(1H-pyrazol-3-yl)ethanol) containing 1 mM NAD\textsuperscript{+}, 200 mM IPA, 0.1 mM PLP, 1 mM FAD in 50 mM potassium phosphate buffer pH 8.0 was prepared. For the single-phase flow reaction, this 10 mM substrate solution was directed into the PBR. The flow rate was 122 μL min\textsuperscript{-1} to allow a residence time of 30 min. For two-phases flow reactions without recirculation, the 10 mM substrate solution was flowed into the PBR at 122 μL min\textsuperscript{-1} to allow a residence time of 15 min with a peristaltic pump. Air was flowed into the PBR at the same flow rate with a second peristaltic pump and mixed to the substrate solution stream using a T-junction. The resulting 50 : 50 substrate solution/air segmented flow was directed into the PBR. Samples were taken from the exiting flow streams of single-phase and two-phases flow systems and analysed by HPLC, as described in Analytical method.
For the two-phases flow reactions with recirculation, a 50 mM substrate solution 5 mM NAD+, 100 mM IPA, 0.1 mM PLP, 1 mM FAD in 50 mM potassium phosphate buffer pH 8.0, and a 30 mM substrate solution 3 mM NAD+, 60 mM IPA, 0.1 mM PLP, 1 mM FAD in 50 mM potassium phosphate buffer pH 8.0, were prepared. 9.151 mL of the 10 mM or the 50 mM substrate solution were flowed at 122 μL min⁻¹ to allow a residence time of 15 min. The exiting flow stream was placed in the same container as the substrate solution.

Air was flowed into the PBR at the same flow rate. After one pass of the system, the flow rate was set up to 122 mL min⁻¹. A sample was taken after 75 min, a sample was taken and analysed by HPLC. Other samples were taken after 2 and 4 (after 2.5 h and 5 h, respectively).

Amines catch-and-release in continuous flow

3 g of Relisorb® HG400SS were resuspended in 30 mL of 20 mM NaOAc. After 2 h incubation at room temperature under mild agitation, the resulting carrier, was filtered and washed with H₂O. Resulting aldehyde groups were quantified as previously described. An Omnifit glass column (6.6 mm i.d. × 100 mm length) was filled with 3 g of this tailor-made aldehyde resin (column volume, 3.66 mL).

The product mixture resulting from the 30 mM flow reaction (implemented with segmented flow and recirculation) was flowed to the system. The flow rate was set up to 122 μL min⁻¹ to allow a residence time of 30 min (temperature off). 4 column volumes of 50 mM phosphate buffer pH 8 were flowed to the system. The elution of the trapped primary amines by the aldehyde groups was carried out with 4 column volumes of 0.2% HCl.

Analytical methods

100 μL of sample with a maximum concentration of substrate (10 mM) were added to 900 μL of MilliQ H₂O. The samples were analysed by HPLC (Accucore™ aQ C18 Polar Endcapped HPLC Column, Thermo Fisher Scientific (2.6 μm, 4.6 × 150 mm), measuring at 210 nm, using an isocratic method with H₂O 0.1%TFA) over 5 minutes with a flow rate of 0.8 mL min⁻¹. The retention times of the different were: 2-(1H-pyrrozol-3-yl)ethanol 3.45 min, and betazole 2.38 min. Molar conversions were calculated through a standard curve of the product.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Prof. Sieber (TU Munich) for kindly providing the plasmid harbouring the LpNOX gene. The authors wish to acknowledge the Biotechnology and Biological Research Council [grant number BB/P002536/1] and H2020 ERACoBioTech programme (project ID: 61 HOMBIOCAT) also funded through the Biotechnology and Biological Sciences Research Council [grant number BB/R021287/1].

Notes and references


