Highly Efficient Oxidation of Amines to Aldehydes with Flow-based Biocatalysis

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A new mild and efficient process for the aqueous preparation of aldehydes, which are employed as flavour and fragrance components in food, beverage, cosmetics, as well as in pharmaceuticals, was developed using a continuous-flow approach based on an immobilised pure transaminase-packed bed reactor. HEWT, an α-transaminase from the haloadapted bacterium Halomonas elongata, has been selected for its excellent stability and substrate scope. Sixteen different amines were rapidly (3–15 min) oxidised to the corresponding aldehydes (90 to 99%) with only 1 to 5 equivalents of sodium pyruvate. The process was fully automated, allowing for the in-line recovery of the pure aldehydes (chemical purity > 99% and isolated yields above 80%), without any further work-up procedure.

Introduction

Aromatic aldehydes are important intermediates in a number of synthetic processes and have a prominent role as flavour and fragrance components. Among other synthetic methods,[1] they can be obtained from the corresponding primary aromatic amines, which are readily available substrates. Methods for the oxidation of amines to carbonyl compounds have received significant attention, but these approaches are frequently poorly sustainable, because they produce waste and by-products that are difficult to recycle, require drastic reaction conditions, and often proceed with poor selectivity.[1a,2]

Biocatalytic processes are interesting alternatives for amine oxidations under mild and benign conditions. For example, copper amine oxidases (CAOs) have been used to catalyse the oxidation of primary amines to aldehydes (while O₂ is simultaneously reduced to H₂O₂).[3] Vanillin has been prepared by oxidation of vanillylamine using an amine oxidase (AO) from Aspergillus niger.[4] Recently, selective oxidation of amines to aldehydes has been obtained using a laccase with TEMPO (2,2,6,6-tetramethylpiperidine N-oxide) as mediator and O₂ as oxidant.[5] Aromatic aldehydes can also be enzymatically prepared using other approaches, such as oxidation of primary alcohols[6] and reduction of carboxylic acids.[7]

In this context, we developed an efficient bio-preparation of nature-identical flavours and fragrances exploiting the immobilised amine transaminase from the moderate halophilic bacterium Halomonas elongata (HEWT),[8] which is able to tolerate a range of temperature, pH, salts and co-solvents in a continuous flow reactor. The combination of biocatalysis and flow reactor technology can be considered as an enabling methodology intrinsically compatible with the principles of green chemistry.[9] Flow-based biocatalysis was recently applied for peptide condensation,[10] hydrolysis and formation of esters and sugars,[11] stereoselective carbonyl reduction,[12] formation of C–C bonds,[13] production of nucleosides,[14] monosaccharides,[15] and oligosaccharides,[16] and interconversion of carboxyls and amines using transaminases.[17]

We recently reported on the application of HEWT in flow for the biosynthesis of amines[18] and we describe here an eco-friendly and scalable process that enhances the oxidising capability of this covalently immobilised enzyme for the production of aldehydes. The products are aromatic aldehydes used as flavours and fragrances in food, beverage, cosmetics and pharmaceuticals. They have been obtained in excellent yields, with unprecedented reaction times if compared with traditional batch methods. The use of pyruvate as amino acceptor is extremely favourable and by-product which it generates, the natural amino acid l-alanine, is completely benign and can be easily recovered. Furthermore, this approach circumvents potential issues often encountered with whole-cell biotransformations, such as generation of debris, swelling and permeability.

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Results and Discussion

Pure HEWT (imm-HEWT) was immobilised on an epoxy-resin as reported by Planchestainer et al.\textsuperscript{[18]} and the supported biocatalyst (5 mg gram\textsuperscript{-1}) was then used in a packed-bed flow reactor. The system was firstly tuned by optimising the preparation of benzaldehyde starting from the corresponding benzylamine (Scheme 1).

To maximise the solubility of the amine, 10% of DMSO was used as co-solvent in the phosphate buffer (50 mM, pH 8.0). The reaction was performed under optimised conditions at 37 °C and atmospheric pressure with just one equivalent of pyruvate, as the equilibrium for this reaction is extremely favourable; complete substrate oxidation (molar conversion > 99%) was obtained with only 3 minutes of residence time (flow rate 0.3 mL min\textsuperscript{-1}).

Notably, the use of the same immobilised enzyme in batch gave a full oxidation in about 2 hours.

The optimised conditions were applied to the bioconversion of different benzylamines into the corresponding flavour aldehydes (Table 1).

Specific reaction rates in the batch and continuous-flow systems were calculated using the equations reported in the Experimental Section; the time taken (conversion rate) for the reaction to reach maximum conversion, whether in batch or continuous-flow, was calculated and normalised to the amount of catalyst used for both set-ups.\textsuperscript{[114]}

Benzylamine-derivatives (entries 1–8) were oxidised into the corresponding aroma-compounds with high molar conversion; in all cases, a greater than 4-fold rate increase was observed if reactions were conducted under flow conditions, as conversions ≥ 90% were reached within a residence time between 3 and 10 minutes (flow rate 0.3 mL min\textsuperscript{-1} and 0.1 mL min\textsuperscript{-1}, respectively), at 37 °C and atmospheric pressure.

The process was implemented with the addition of an in-line acidification step followed by extraction with EtOAc. The two phases were continuously separated using a Zaiput liquid/liquid separator and the desired aldehydes were recovered in the organic phase, significantly accelerating the overall work-up, as no further purification was required (Scheme 2).

This protocol was successfully applied to substrates 1a–1h. Aldehydes obtained from substrates 1i and 1j (entries 9 and 10) proved initially difficult to recover as they were retained by the packing material, despite various and extensive washing steps.

A second set of amines (1k–1p) was investigated using the same methodologies (either in a monophasic environment or the biphasic one) to prove the versatility of the system with different aromatic substrates. (Table 2).

The batch oxidation of the tested (aryl)alkyl amines with methyl/ethyl side chain (entries 11–14) allowed for the preparation of flavour aldehydes 2k (hyacinth note), 2j (floral note), 2m (floral note), and 2n (violet note) with excellent conversion (> 99%), although the reactions required several hours to go to completion. In line with our observations for the benzylamine derivatives, the same molar conversion was obtained within 3 to 15 minutes of residence time in flow, thus strongly increasing the overall productivity. In particular, piperonylamine (1n) was successfully converted into the corresponding aldehyde (piperonal 2n, the violet fragrance, also known as heliotropin) in only 15 minutes (14-fold faster reaction rate) with > 99% conversion at 45 °C, demonstrating the good stability and adaptability of this enzyme also at higher temperatures. Both (S) and (R)-2-phenyl-1-propylamine (1l and 1m, respec-
The enzyme equally converted both enantiomers and did not show any stereopreference for this particular molecule (entries 12 and 13).

However, the oxidation of cinnamylamine (1a, entry 15) to cinnaldehyde (2a, cinnamon aroma) and hydrocinnamylamine (1p, entry 16) to hydrocinnamaldehyde (2p, honey aroma), appeared more challenging. The batch reaction with an equimolar concentration of amino donor resulted in poor conversion after 24 hours (50 and 52%), without any significant increase over a longer incubation time, likely owing to an unfavourable equilibrium. Under flow conditions, with one equivalent of pyruvate, the conversions achieved were 50% and 25% respectively, despite increasing the residence time to 30 min. To displace the equilibrium, the concentration of pyruvate was increased to 2 and 5 equivalents with respect to the aldehydes 1a and 1p, yielding 95% of cinnamaldehyde and

| Entry | Substrate | Reaction time [min] | M. c. [%] | Conv. Rate [μmol min⁻¹ g⁻¹] | Residence time [min] | M. c. [%] | Conv. Rate [μmol min⁻¹ g⁻¹] |
|-------|-----------|---------------------|----------|----------------------------|---------------------|----------|----------------------------|
| 1     | 1a        | 120                 | > 99     | 0.83                       | 3                   | > 99     | 4.24                       |
| 2     | 1b        | 120                 | > 99     | 0.83                       | 3                   | > 99     | 4.24                       |
| 3     | 1c        | 120                 | > 99     | 0.83                       | 3                   | > 99     | 4.24                       |
| 4     | 1d        | 120                 | > 99     | 0.83                       | 3                   | > 99     | 4.24                       |
| 5     | 1e        | 120                 | > 99     | 0.33                       | 10                  | > 99     | 1.41                       |
| 6     | 1f        | 300                 | > 99     | 0.33                       | 10                  | 90       | 1.29                       |
| 7     | 1g        | 300                 | > 99     | 0.33                       | 10                  | 90       | 1.29                       |
| 8     | 1h        | 120                 | > 99     | 0.83                       | 3                   | 95       | 4.07                       |
| 9     | 1i        | 300                 | > 99     | 0.33                       | 10                  | > 99[c] | 1.41[c]                   |
| 10    | 1j        | 300                 | > 99     | 0.33                       | 10                  | > 99[c] | 1.41[c]                   |

[a] Reactions were performed in the presence of 10 mM substrates and pyruvate, 0.1 mM PLP, 10% DMSO was used as co-solvent at 37 °C. Isolated yields are reported in the Experimental Section. [b] Conversion rates are normalised to the amount of enzyme used in the reaction and calculated as reported in Ref. [11a]. [c] Liquid-liquid-phase flow stream (see procedure summarised in Scheme 3), in this case DMSO was not added to the buffer.
90% of the saturated aldehyde with 15 minutes of residence time at 45 °C. This result underlines the fact that process control strategies (in our case, the optimisation of stoichiometric ratio of the substrates) help to maximise the productivity of HEWT by accelerating the reaction, while shifting the equilibrium to the product side.

Conclusions

A new biocatalytic method for the synthesis of aldehydes with extensive applications as components of flavours and fragrances was developed. This is the first example of a transaminase exploited in a flow chemistry reactor under highly favourable oxidising conditions for the preparation of aromatic aldehydes, showing excellent adaptability and stability during the processes. The use of a flow-based approach allowed for dramatic accelerations of the reactions, with isolated yields above 80% and very short residence times (3–15 min) of the substrates. This system required, in the majority of cases, only one equivalent of pyruvate as the amino acceptor, which leads to the formation of L-alanine as by-product. A successful implementation was achieved with an in-line extraction step, which permitted the recovery of the desired pure aldehydes in the organic stream and L-alanine in the aqueous one, with an extremely simplified work-up procedure and almost no manipulation. As a result of the high local concentration of the (bio)catalyst and the enhanced heat and mass transfer, the combination between biocatalysis and flow chemistry reactors not only leads to significant reductions of reaction times and increased productivity, but it can be also considered a sustainable technology for the production of aldehydes commonly used in food, cosmetic, and pharmaceutical industry.

Experimental Section

Expression, purification, and immobilisation of HEWT in E. coli

Protein expression and purification was performed following previously reported protocols in Cerioli et al.; immobilisation was conducted according to the procedure reported by Planchestainer et al.

Batch reactions with immobilised HEWT

Batch reactions using the imm-HEWT were performed in 1.5 mL micro centrifuge tubes; 500 μL reaction mixture in 50 mM phosphate buffer pH 8.0, containing 10 mM pyruvate, 10 mM amino donor substrate, 0.1 mM PLP, and 50 mg of imm-HEWT (5 mg mL⁻¹) was left under gentle shaking at 37 °C. 10 μL aliquots were quenched with trifluoroacetic acid (TFA) 0.2% every hour and then analysed by HPLC equipped with a Supelco LC-18-T column (250 mm × 4.6 mm, 5 μm particle size; Supelco, Sigma–Aldrich, Germany).

Table 2. Preparation of aryl-alkyl aldehydes from the corresponding amines

| Entry | Substrate | Reaction time [min] | M.c. [%] | Conv. Rate [μmol min⁻¹ g⁻¹] | Residence time [min] | M.c. [%] | Conv. Rate [μmol min⁻¹ g⁻¹] |
|-------|-----------|---------------------|---------|-----------------------------|----------------------|---------|-----------------------------|
| 11    |           | 120                 | >99     | 0.83                        | 3                    | >99     | 4.24                        |
| 12    |           | 180                 | >99     | 0.55                        | 3                    | >99     | 4.24                        |
| 13    |           | 180                 | >99     | 0.55                        | 3                    | >99     | 4.24                        |
| 14    |           | 1440                | >99     | 0.07                        | 15                   | >99     | 0.95                        |
| 15    |           | 1440                | 50      | 0.04                        | 15                   | >99     | 1.02                        |
| 16    |           | 300                 | 24      | 0.04                        | 15                   | 90      | 0.86                        |

[a] Reactions were performed in the presence of 10 mM substrates and pyruvate, 0.1 mM PLP, 10% DMSO was used as co-solvent at 37 °C. Isolated yields are reported in the Experimental Section. [b] Conversion rates are normalised to the amount of enzyme used in the reaction and calculated as reported in Ref. [11a]. [c] Liquid-liquid-phase flow stream (see procedure summarised in Scheme 3), in this case DMSO was not added to the buffer. [d] Reactions performed at 45 °C. [e] 20 mM Pyruvate. [f] 50 mM pyruvate. [g] Calculated at a similar degree of conversion of the batch reaction.
Flow reactions with immobilised HEWT

Continuous flow biotransformations were performed using a R2+/R4 Vapourtec flow reactor equipped with an Omnifit glass column (0.3421 mm i.d. × 100 mm length) filled with 0.7 g of imm-HEWT (5 mg g⁻¹). A 20 mM sodium pyruvate in phosphate buffer (50 mM, pH 8.0) containing 0.1 mM PLP and 20 mM amino donor solution with 10% of DMSO were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 1.0 mL). The flow rate was varied and optimised. An in-line acidification was performed by using an inlet of 1 N HCl aqueous solution (flow rate: 0.1 mL min⁻¹) that was mixed to the exiting reaction flow stream using a T-junction. The resulting aqueous phase was extracted in-line using a stream of EtOAc (flow rate: 0.2 mL min⁻¹) and a Zaiput liquid/liquid separator. Both the organic and aqueous phase were analysed by HPLC using the above reported conditions. The amount of substrate and product was evaluated by exploiting a previously prepared calibration curve. For the optimisation procedure, the reactions have been performed by injecting 250 μL of each starting solutions (volume of EtOAc used for the in-line extraction: 1 mL). To isolate the product, 10 mL of aqueous solution (flow rate: 0.1 mL min⁻¹) containing 0.1 mM pyruvate in phosphate buffer (50 mM, pH 8.0) was used (volume of EtOAc used for the in-line extraction: 1 mL). The organic phase, containing the aldehydes, was evaporated to yield the aldehydes.

Characterisation of the products

The purity of aldehydes was assessed by HPLC and ¹H NMR. ¹H NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are expressed in Hz.

**Benzaldehyde (2a):** colourless oil; yield 95%; ¹H NMR (CDCl₃) δ = 10.00 (s, 1H), 8.15–8.12 (m, 2H), 7.67–7.51 ppm (m, 3H).

**p-Tolualdehyde (2b):** yellow oil; yield 96%; ¹H NMR (CDCl₃) δ = 9.95 (s, 1H), 7.74 (d, J = 7.5 Hz, 2H), 7.32 (d, J = 7.5 Hz, 2H), 2.40 ppm (s, 3H).

**Anisaldehyde (2c):** colourless oil; yield 94%; ¹H NMR (CDCl₃) δ = 9.85 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 6.96 (d, J = 8.0 Hz, 2H), 3.90 ppm (s, 3H).

**Ethyl benzoate (2d):** yellow oil; yield 94%; ¹H NMR (CDCl₃) δ = 9.98 (s, 1H), 7.81 (d, J = 8.1 Hz, 2H), 7.36 (d, J = 8.1 Hz, 2H), 2.74 ppm (q, J = 7.5 Hz, 2H), 1.27 ppm (t, J = 7.5 Hz, 3H).

**Hydroxybenzaldehyde (2e):** yellow oil; yield 92%; ¹H NMR (CDCl₃) δ = 9.85 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 6.73 ppm (d, J = 8.3 Hz, 2H).

**Cuminaldehyde (2f):** colourless oil; yield 84%; ¹H NMR (CDCl₃) δ = 9.98 (s, 1H), 7.84 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 3.00 ppm (septet, J = 6.5 Hz, 1H), 1.30 ppm (d, J = 6.9 Hz, 6H).

**Salicylaldehyde (2g):** yellow oil; yield 82%; ¹H NMR (CDCl₃) δ = 11.00 (bs, 1H, OH), 9.85 (s, 1H), 7.46–7.54 (m, 2H), 6.94–7.00 ppm (m, 2H).

**Vanillin (2h):** white solid; yield 90%; ¹H NMR (CDCl₃) δ = 9.78 (s, 1H), 7.37–7.40 (m, 2H), 7.02 (d, J = 8.5 Hz, 1H), 6.72 (bs, 1H, OH), 3.90 ppm (s, 3H).

**Veratraldehyde (2i):** yellow solid; yield 96%; ¹H NMR (CDCl₃) δ = 9.85 (s, 1H), 6.70–7.65 (m, 3H), 3.98 (s, 3H), 3.95 ppm (s, 3H).

**Syringaldehyde (2j):** yellow solid; yield 94%; ¹H NMR (CDCl₃) δ = 9.83 (s, 1H), 7.15 (s, 2H), 6.10 (s, 1H), 3.98 ppm (s, 6H).
Phenylacetaldehyde (2k): pale yellow oil; yield 97%; 1H NMR (CDCl3) δ = 9.70 (t, J = 2.0 Hz, 1H), 7.30–7.10 (m, 5H), 3.56 ppm (d, J = 2.0 Hz, 2H).

2-Phenylpropanaldehyde (2l/2m): colourless oil; yield 90%; 1H NMR (CDCl3) δ = 9.62 (s, 1H), 7.30–7.40 (m, 2H), 7.20–7.28 (m, 3H), 3.60 (q, J = 7.0, 1H), 1.45 ppm (d, J = 7.0, 3H).

Piperonal (2n): white solid; yield 87%; 1H NMR (CDCl3) δ = 9.80 (s, 1H), 7.10, (dd, J = 7.9 Hz, 1H), 7.32 (d, J = 7.6 Hz, 1H), 6.92 (d, J = 7.9 Hz, 1H), 6.07 ppm (s, 2H).

Trans-Cinnamaldehyde (2o): yellow oil; yield 89% 1H NMR (CDCl3) δ = 9.70 (d, J = 7.7 Hz 1H), 7.55 (dd, J = 5.2, 2.0 Hz, 2H), 7.50 (d, J = 15.9 Hz, 1H), 7.42–7.46 (m, 3H), 6.73 ppm (dd, J = 15.9, 7.7 Hz, 1H).

Hydrocinnamaldehyde (2p): pale yellow oil; yield 86% 1H NMR (CDCl3) δ = 9.76 (s, 1H), 7.35 (q, J = 7.4 Hz, 2H), 7.25–7.30 (m, 3H), 3.00 (t, J = 15.1 Hz, 2H), 2.82–2.85 ppm (m, 2H).

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Conflict of interest

The authors declare no conflict of interest.

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