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Continuous flow biocatalysis: production and in-line purification of amines by immobilised transaminase from *Halomonas elongata*

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The continuous flow synthesis of a series of amines was successfully achieved by exploiting the enhanced stability and broad substrate scope of an immobilised transaminases from *Halomonas elongata* (HEWT). A series of substrates were tested in flow reactors and transformed to the corresponding amines in good to excellent yields. The process was implemented with an integrated in-line purification step for the recovery of the pure amines.

Organic chemistry is constantly evolving to fulfil the ever high demand for fine chemicals, such as natural products or Active Pharmaceutical Ingredients (APIs). In the last two decades, the introduction of enzymes as novel natural catalyst has led to a new concept for industrial processes where biotransformations have substituted a range of traditional synthetic steps generating "greener" routes for the production of pharmaceuticals and agrochemicals.[1–3] In parallel, chemical engineering has significantly changed how reactions are set up and new, more efficient, strategies are now preferable to conventional batch reactors.[4,5] In this panorama, flow chemistry represents a powerful instrument to increase production in shorter reaction times, making the process economically more attractive. It is not surprising that flow processes have been booming in the last few years.[6] More recently, innovative flow-bioreactions have combined the flow-chemistry's throughput to the catalytic power of enzymes.[7-10] The merging of these two modern approaches in a single-enzymatic reaction process simulates the extremely efficient cells' metabolic pathways.[11] However, there are still only few examples of flow enzymatic reactions, especially if cofactor-depended enzymes are involved.[12,13]

We developed an efficient bioproduction of amines exploiting the immobilised (*S*)-selective amine transaminase from *Halomonas elongata* (HEWT), which showed a very broad substrate scope, tolerating a range of temperature, pH, salt, and co-solvents,[14] in a continuous flow-reactor. Transaminases play a central role in the biocatalytic preparation of enantiopure amines and amino acids; challenging reactions to achieve by conventional synthesis.[15] They are gaining attention because of their application in the manufacturing of pharmaceuticals intermediates on a large scale.[16,17] Recently, Andrade *et al.* developed an immobilised whole cell system expressing a transaminase for the synthesis in flow of amines in excellent yields.[18] However such system works efficiently only at extremely low flow (0.02 mL/min) requiring days to convert reasonable volumes of reactions due to the minimal catalyst loading (120mL in 5 days). Furthermore, the cell integrity is preserved only by running the reaction in 100% MTBE and the cell permeability to a range of substrates may be challenging. In the present paper, we introduce the first example of flow reaction for the synthesis of different amines exploiting the covalently immobilised, cell-free, amino transaminase from *H. elongata*, bypassing the potential issues posed by whole-cell systems, with an excellent lifespan of the immobilised catalysts, fast reaction times due to very high and localised catalyst load, and excellent isolated yields facilitated by an in-line liquid-liquid extraction system.

Covalent immobilisation of single enzymes is widely applied to significantly enhance the protein stability to high temperatures, organic solvents, and extreme pHs.[19] Furthermore, it allows for an easier recovery and reutilization of the biocatalysts and its incorporation in flow reactors.[20,21] In this study, the immobilisation strategy previously successfully applied to His-HLADH-EE[22] and HvADH2[23] was adapted to stabilise HEWT, since its quaternary structure is quite unstable. The methodology, originally developed by Guisan et al.,[24]

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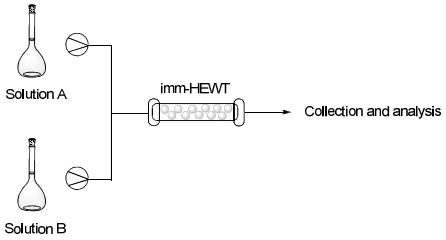
exploits the poly-His-tag fused to the enzyme for a selective interaction with a metal derivatised epoxy-resin.[25] The immobilised HEWT (imm-HEWT) was subsequently applied to continuous-flow reactions maintaining the biocatalyst in a packed-bed reactor to decrease reaction times and increase the productivity. The addition of an in-line purification step allows for the recovery of the pure products without any additional work-up procedure.

Quaglia *et al.*[22] found that Cobalt (II) was the best metal ion for complexation with His-HLADH-EE and screening of a selection of metals for HEWT immobilization led to the same conclusion (Fig. S.1). Optimal recovered activity was achieved at 24 h incubation time (1.8 U/g, approximately 30% compared to the free enzyme), and longer times did not increase this further. Temperature had a detrimental effect on the procedure, with the recovered activity dropping to 6% if the immobilisation was carried out at 37 °C. This is in agreement with what previously reported for His-HLADH-EE.[22] Upon immobilisation, the biocatalyst showed enhanced stability with respect to the free form (nearly 90% preserved activity after storage in buffer for 30 days at 4 °C, pH 8) as well as a marked increased tolerance to organic solvents (Fig. S.2) and reusability (Fig. S.3). The imm-HEWT shows also a slightly higher thermal stability when incubated at 40 to 50 °C, while pH optimum and stability are unchanged. The optimised immobilisation protocol was also applied directly to the crude cell extract. Interestingly, this led to a better recovery of activity than with the pre-purified enzyme. The activity on the beads was consistently 3.5 U/g, 40% retained activity compared to the free enzyme and a 10 % increase in recovered activity with respect to the pre-purified preparation. This result was confirmed by swapping Ni^{2+} for Co^{2+} in the protocol and additional investigation showed a beneficial stabilizing effect of the endogenous proteins on the overall process (Fig. S.4).

The imm-HEWT catalysed continuous-flow reaction was firstly tuned exploiting as model reaction the conversion of (*S*)-(−)-1-phenylethylamine in acetophenone in the presence of pyruvate (Scheme 1). The reaction was performed at 37 °C, at atmospheric pressure, and different loadings of the immobilised enzyme (*i.e.*, 0.5, 1.0, 2.0, 5.0 mg of enzyme per gram of resin, 3 minutes of residence time) were tested, resulting in a substrate conversion of 38%, 76%, 95%, and >99%, respectively. The resin, with an enzyme load of 5 mg/g_{resin}, allowed for the complete substrate conversion within only 1 minute of residence time and, for this reason, it was selected for the subsequent investigations (for details see the SI). Notably, the use of the same resin in batch (50 mg resin in 0.5 mL reaction volume) gave a full conversion in approximately 180 minutes. The stability of the biocatalyst under continuous flow was assessed by performing the biotransformation under the conditions reported in Table 1 entry 1. Samples were collected every 5 minutes (3.9 mL of solution, flow 0.78 mL/min), analysed by HPLC and the consistency of the results obtained over time was verified in terms of conversion, showing excellent stability under the chosen working conditions. A total volume of about 250 mL was collected in just over 5 hours. Full conversion (>99%) was observed in the first 13 fractions (about 50 mL of resulting solution). Then, the conversion slowly decreased, reaching 80% in the last collected fraction. This allowed for the pre-packed column to be utilised extensively with different reaction batches without any considerable loss of activity.



Scheme 1. Solution A: 50 mM solution of (*S*)-(−)-1-phenyl-ethylamine in phosphate buffer (50 mM, pH 8.0) containing 5% DMSO. Solution B: 20 mM solution of pyruvate containing 0.1 mM PLP. T = 37 °C, P = atm.

The success of the first test reaction led to a series of alternative and more challenging substrates (aldehydes) for their biotransformation into amines in flow using L-alanine as amino donor (Table 1). *p*-NO_2-benzaldehyde was completely converted into the correspondent *p*-NO_2-benzylamine within only 2 minutes of residence time at 37 °C and atmospheric pressure. To ameliorate the solubility of *p*-NO_2-benzaldehyde, the substrate concentration was lowered to 10 mM and DMSO was increased to 10%. The process was then implemented by adding an in-line purification step consisting of an in-line basification to pH 11, followed by an extraction with EtOAc (Scheme S.1). Any trace of unreacted aldehyde that could be present in the organic phase (< 1%) was trapped by directing the flow stream into a column packed with polymer supported benzylamine. This strategy allowed for the easy recovery of the desired amine (95% isolated yield) by simply evaporating the organic solvent, significantly simplifying and accelerating the work-up procedures.

Cinnamylamine, a useful starting material for the synthesis of biologically active molecules,[26] was easily synthetized from cinnamaldehyde (90% conversion) within 2 minutes (Table 1, entry 3) using the otpimised procedure. The same reaction in batch reaches 90% conversion in 24 hours. Applying the in-line purification step described above, we performed the reaction in flow on 50 mL of 20 mM substrate solution and we isolated the pure product (*i.e.*, cinnamylamine) in 86% yield in 2 minutes of residence time.

The amination of vanillin yields vanillylamine which is a valuable building block for the synthesis of natural products, such as capsaicinoids.[27] However, the reaction in batch underwent poor conversion (18%) in 24 hours without significant improvement over longer incubation time. Under flow conditions, 18% conversion was achieved in 2 minutes of residence time at 37 °C. To increase the conversion, the effect of different parameters, *i.e.*, temperature (range 37-60 °C), residence time (range 3-120 min), and pressure (range atmospheric-250 psi) were evaluated without significant success. A recycling system of unreacted vanillin was therefore put in place following product removal by exploiting an acidic resin (A15). The unreacted substrate was recycled by mixing it with the amine donor and flowing the stream through the imm-HEWT packed column (Scheme S.2), to be converted into vanillylamine. Exploiting this strategy, a 50% conversion was obtained after 5 consecutive cycles. A further increase of the number of cycles did not lead to an increase of the conversion. A possible explanation can be the high Km for vanillin that leads to low reaction rate at low

Table 1. Amines bioproduction summary where batch reaction and flow reactions molar conversion (m.c.), reaction time, and conversion per unit time normalised for the amount of enzyme are reported. Results are the average of three trials with standard deviations lower than 5%.

			Batch			Continuous flow		
Entry	**Substrate** (10 mM)	**Amino donor**	**Conversion time**	**m.c.**	**µmol min^{-1} g^{-1}**	**Residence time**	**m.c.**	**µmol min^{-1} g^{-1}**
1	Pyruvate	(*S*)-(−)-1-phenylethylamine (25 mM)[a]	180 min	>99%	0.54[d]	1 min	>99%	7.8[d]
2	*p*-NO_2-Benzaldehyde[b,c]	L-Alanine (500 mM)	210 min	>99%	0.24[d]	2 min	>99%	1.9[d]
3	Cinnamaldehyde[b]	L-Alanine (500 mM)	24 h	92%	0.06[d]	2 min	90%	3.5[d]
4	Vanillin[b]	L-Alanine (500 mM)	24 h	18%	0.012[d]	2 min	18%	0.7[d]
5	Vanillin[b]	L-Alanine (500 mM)	24 h	18%	0.012[d]	5 x 2 min	50%	0.4[d]

[a] 5 % DMSO used as co-solvent

[b] 10 % DMSO used as co-solvent

[c] Substrate concentration: 5 mM

[d] Calculated as reported in ref. 9

substrate concentration. The vanillylamine was then recovered as vanillylamine hydrochloride from the A15 resin by flushing the column with a diluted solution of HCl with an isolated yield of 46%.

In conclusion, amine transaminase from *Halomonas elongata* (HEWT) was successfully covalently immobilised onto metal-derivatised epoxy Sepabeads. The immobilisation of the enzyme retained 30% to 40% of the original activity and increased organic solvent tolerance. Furthermore, it allowed for an easy catalyst recovery and activity was unchanged when assessed over several cycles. A continuous production of amines was performed for the first time exploiting the emerging combination of an immobilized, cell-free, biocatalyst and continuous flow reactors, leading to the reduction of reaction time by 1 to 2 orders of magnitude, due to the very high catalyst loading achievable, as well as an increased productivity of the process compared with batch mode. The high efficiency of heat and especially mass transfer in a flow system allows to speed up the bioconversion rate with respect to the batch system, as demonstrated also for continuous flow chemical processes.[28] The application of flow reactor technology to biocatalysis improves the overall process in terms of efficiency (rates and yields), reuse of the biocatalyst (which is not strained by mechanical stirring or agitation), easy and intensified online downstream processes (recovery and purification);[29,30,31] hence, integration of flow chemistry and biocatalysis can be considered as a key technology intrinsically compatible with the principle of green chemistry.

Notes and references

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