

Article

Efficient Amino Donor Recycling in Amination Reactions: Development of a New Alanine Dehydrogenase in Continuous Flow and Dialysis Membrane Reactors

David Roura Padrosa ^{1,2} , Zoya Nisar ² and Francesca Paradisi ^{1,2,*} 
¹ Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland; david.roura@dcb.unibe.ch

² School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK; zoyanisar@outlook.com

* Correspondence: francesca.paradisi@dcb.unibe.ch; Tel.: +41-(0)-316-314-002

Abstract: Transaminases have arisen as one of the main biocatalysts for amine production but despite their many advantages, their stability is still a concern for widespread application. One of the reasons for their instability is the need to use an excess of the amino donor when trying to synthesise amines with unfavourable equilibria. To circumvent this, recycling systems for the amino donor, such as amino acid dehydrogenases or aldolases, have proved useful to push the equilibria while avoiding high amino donor concentrations. In this work, we report the use of a new alanine dehydrogenase from the halotolerant bacteria *Halomonas elongata* which exhibits excellent stability to different cosolvents, combined with the well characterised CbFDH as a recycling system of L-alanine for the amination of three model substrates with unfavourable equilibria. In a step forward, the amino donor recycling system has been co-immobilised and used in flow with success as well as re-used as a dialysis enclosed system for the amination of an aromatic aldehyde.

Keywords: enzyme immobilization; transaminase; amino acid dehydrogenase; flow biocatalysis



Citation: Roura Padrosa, D.; Nisar, Z.; Paradisi, F. Efficient Amino Donor Recycling in Amination Reactions: Development of a New Alanine Dehydrogenase in Continuous Flow and Dialysis Membrane Reactors. *Catalysts* **2021**, *11*, 520. <https://doi.org/10.3390/catal11040520>

Academic Editor: Juan M. Bolivar

Received: 23 March 2021

Accepted: 15 April 2021

Published: 20 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Amines are one of the most important building blocks in the synthesis of pharmaceuticals, agrochemicals, and food additives. This ubiquity in commercial products, has led to an increased interest in creating more cost-efficient and “greener” strategies for their synthesis. In this sense, the use of enzymes as catalysts has provided significant advance in the sustainable synthesis of amines [1–4] and transaminases are primarily employed. Briefly, transaminases are a group of pyridoxal-5'-phosphate (PLP) dependant enzymes capable of transferring an amino group from an amino donor molecule (i.e., an amino acid) to an amino acceptor carbonyl group. This reaction is achieved through two half-reactions, where the amino group is first transferred into the PLP initially bound to the enzyme to form pyridoxamine-5'-phosphate (PMP), which then reacts with the amino acceptor to form the final product and recover the coenzyme in its initial state, bound to the protein [5].

Although transaminases have already been implemented in the synthesis on large scale of important molecules such as Sitagliptin [6], aminotetraline, and acetophenone [7], their broad application is still hampered, specially, by their stability [8,9]. The loss of stability over time is attributable to the loss of the aminated cofactor after the first half-reaction is completed. This has two main plausible reasons: the binding affinity of the cofactor to the enzyme [10] and the presence of an excess of amino donor [11], commonly used to push the amination reaction for substrates with unfavourable equilibria.

To overcome the equilibrium limitation, several strategies have been proposed. The use of isopropyl amine (IPA) as the amino donor produces acetone, which can be easily removed by evaporation from the reaction bulk, forcing the reaction into the amination

direction [12,13]. But IPA can significantly affect the stability of the enzyme [14], introducing a new challenge—the presence of high amount of organics—into the reaction design. Similar problems arise when using S-methylbenzylamine (SMBA), o-xylylene diamine, or 2-(4-nitrophenyl)ethan-1-amine as the amino donors. Both shift the equilibria significantly, SMBA by producing a product which is not normally accepted by the transaminase for the reverse reaction and the other by effectively removing it from the system via polymerisation to create a black precipitate [15] or via tautomerisation to create a red precipitate [16]. These last two methods proved efficient for screening purposes but have not been exploited in synthesis. As an alternative, terminal diamines such as cadaverine (1,4-diaminopentane) or putrescine (1,4-diaminobutane) have also been used to push the equilibria. After deamination, in aqueous solutions, they cyclise forming an imine, pushing the equilibria [4]. With these type of molecules though, most tested ω -transaminase exhibit low activities [17,18]. At last, another strategy is the use of multi-enzymatic systems, that even if more complex, can be advantageous due to their versatility. One of the most common strategies is the use of amino acid dehydrogenases for the recycling of frequent amino donors such as alanine [19–21]. Although they have promising results, amino acid dehydrogenases are cofactor dependant, which normally impacts the cost-effectiveness of the reaction. This can be solved by incorporating a recycling system for the cofactor [22,23]. Moreover, immobilisation of the enzymes allows their reuse, further increasing the cost-effectiveness of the reaction. In this sense, immobilisation of transaminases has been applied extensively to allow the process intensification [24–26]. In these examples though, no recycling system was applied to affect the equilibria and, when needed, an excess of amino donor was used to achieve satisfactory conversions. On the other hand, another approach for biocatalyst recycling consists in its confinement inside a membrane. In this case, instead of covalently immobilising the enzyme, which normally causes a loss of activity due to increased rigidification, the enzymatic cocktail is placed inside a dialysis membrane. This allows the free flow of the substrate and product in and out of the membrane but avoids the loss of enzyme to the reaction bulk, allowing its reuse in subsequent reactions, even in continuous using membrane reactors [27,28].

In this work, the amination of substrates with unfavourable equilibria with two transaminases, CvTA (*Chromobacterium violaceum* transaminase) [29] and HeWT (*Halomonas elongata* ω -transaminase) [30], was targeted. A new alanine dehydrogenase from the halo-tolerant bacteria *H. elongata* (HeAlaDH) was cloned and characterised to be incorporated as a recycling system for alanine. The selection of *H. elongata* as a source for the AlaDH could in fact provide a highly compatible recycling system when this is coupled with HeWT. The new HeAlaDH was successfully coupled with both transaminases and NADH recycling system (formate dehydrogenase from *Candida boidinii*—CbFDH) [31] with three model substrates: cinnamaldehyde, vanillin, and cyclohexanone. To increase the reaction productivity and sustainability, two strategies were tested: the use of immobilised enzymes as packed bed reactors in continuous flow and as a soluble enzymatic cocktail enclosed in dialysis membranes.

2. Results

A gene coding for an alanine dehydrogenase (helo_3819) was identified in the genome of *Halomonas elongata* DSM 2581. This was cloned into a pRSETb vector, expressed in *E. coli* BL21(DE3) and purified by IMAC as a hexamer (confirmed by size exclusion chromatography). The new enzyme exhibited a high specificity for pyruvate as the main substrate, with very good resistance to organic solvents and basic pH (Figures S1–S4). HeAlaDH retained at least 50% of the activity with all tested cosolvents at 10% (*v/v*) and exhibited good resistance to 20% acetonitrile, methanol, and ethanol over 48 h. As for the pH, only acidic (pH < 5) conditions seemed to have a negative effect on the enzyme stability. Overall, these characteristics make it a promising candidate for the coupling with transamination reactions.

2.1. Batch Amination of Unfavourable Carbonyl Acceptors

To assess the suitability of HeAlaDH as an amino donor recycling system, coupled reactions with two different transaminases, CvTA and HeWT [30], were tested in batch with two aromatic aldehydes (cinnamyl aldehyde and vanillin) as well as a cyclic ketone (cyclohexanone). Previous results with equimolar SMBA as the amino donor yielded ~30% cyclohexylamine and 45% vanillyl amine, whereas cinnamaldehyde conversion was better with a 60% conversion over 24 h. The modest obtained conversions were explained by an unfavourable reaction equilibria [30], making them ideal substrates to test our system. Moreover, it is important to note that these substrates required at least 10% dimethyl sulfoxide (DMSO) as co-solvent to be completely solubilised even at the 10 mM scale, highlighting the importance of the co-solvent resistance required by the recycling system.

As no data was available in the literature for the conversion of these substrates with L-alanine as the amino donor, control reactions without any recycling system were also tested (Figure 1). Vanillin was assayed with 50, 5, and 1 eq. of L-alanine as amino donor (Figure S5). In the control, only ~50% of molar conversion was achieved over 24 h despite the use of concentrations of L-Ala up to 500 mM, with no significant conversion after 2 h. On the other hand, when the reaction was coupled with HeAlaDH and CbFDH, with just 1 eq. of the amino donor and 0.1 eq. of the cofactor, HeWT was able to aminate all vanillin at the 10 mM in only 2 h (Figure 1). For cinnamyl aldehyde, the reaction could be enhanced even further. With 1 eq. of L-alanine, HeWT alone was only able to convert 10% of the substrate while in the presence of the recycling system it reached completion within 2 h. The same conversion could be obtained even when reducing the L-Ala to just 0.1 eq. The cyclohexanone/L-Ala pair was significantly worse than the cyclohexanone/SMBA, yielding only 5% of the product in the control system even with 10 eq. of the amino donor. However, the addition of the recycling system pushed the reaction to complete conversion in 24 h. When 1 eq. of amino donor was tried with the recycling system, the conversion was still significant, reaching >80% after one day.

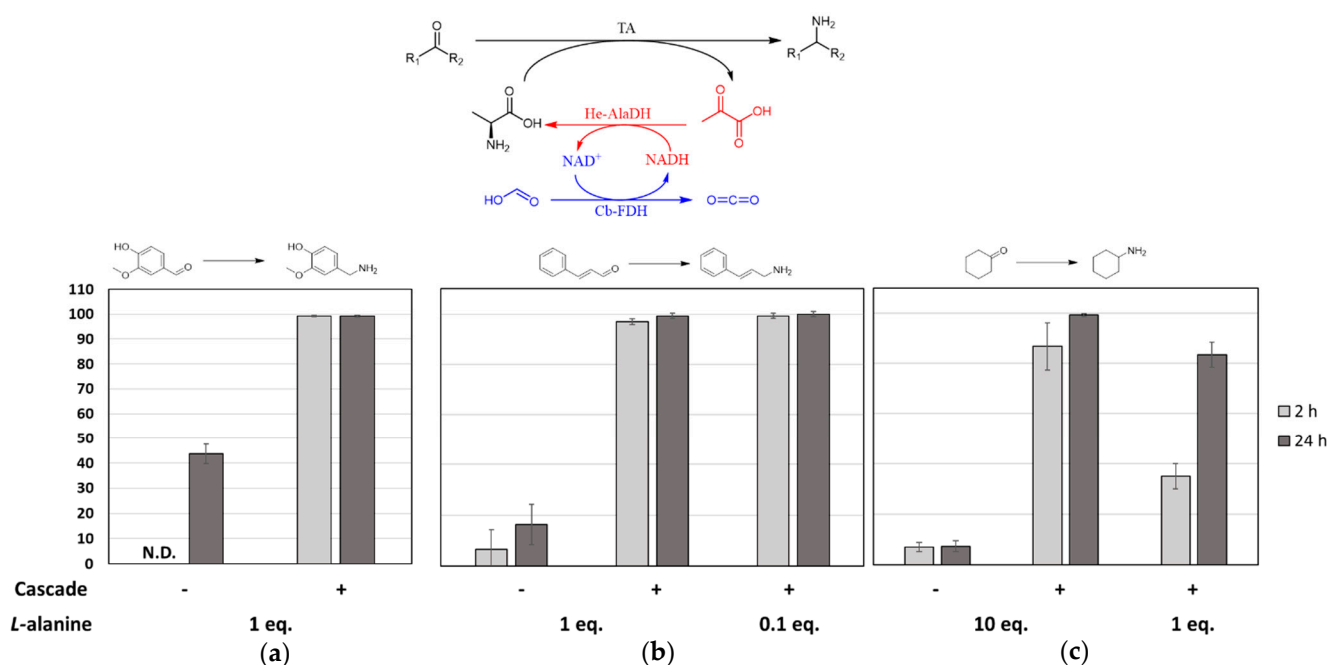


Figure 1. Conversions are expressed as % of molar conversion. The reaction performed with HeWT alone is indicated with a “–” while the presence of the recycling system (HeAlaDH and CbFDH) is indicated with a “+”. Reactions contained 2 mg/mL of HeWT, 0.5 mg/mL of HeAlaDH, 1 mg/mL of CbFDH with 10 mM of the corresponding aldehyde or ketone, 1 mM NAD⁺, 100 mM of ammonium formate, and the desired concentration of alanine in 100 mM phosphate buffer pH 8. N.D. refers to not detected. (a) Vanillin (10 mM) conversion in batch with different amounts of alanine. (b) Cinnamaldehyde amination with equimolar and sub-stoichiometric amount of the L-alanine as the amino donor. (c) Amination of 10 mM cyclohexanone with 10 equivalents or 1 equivalent of L-alanine.

To better understand the contribution of each of the components in the cascade, the reaction rate of these biotransformations was further studied, with both HeWT and CvTA and vanillin as the amino acceptor. The effect of the substrate concentration (at 10 and 50 mM) was examined (Figure 2 and Figure S6). Interestingly, the results indicate that the contribution of HeAlaDH alone is negligible when using the equimolar amount of alanine and cofactor. Only when the whole system was assembled, thus maintaining the pool of reduced cofactor, significant increase in the reaction rate were achieved. As for the effect of the substrate concentration, the reaction rate increased in both cases at the higher scale. This fact could be attributed to an increased capacity of HeAlaDH for pyruvate recycling into alanine and for the transaminase to accept the amino donor. Despite the increased reaction rate, at the higher scale, the maximum molar conversion of the substrate was reached after 72 h (70% for HeWT and 86% for CvTA). In addition, using 0.2 eq. of alanine with 50 mM of the aldehyde substrate, slightly decreased the reaction rate. This suggests a higher dependency of HeWT to the alanine concentration, as the reaction rate was similar to the one at 10 mM scale. In contrast, for CvTA, the reaction rate was only slightly decreased (0.83 ± 0.13 compared to 1.36 ± 0.39 mmols/min).

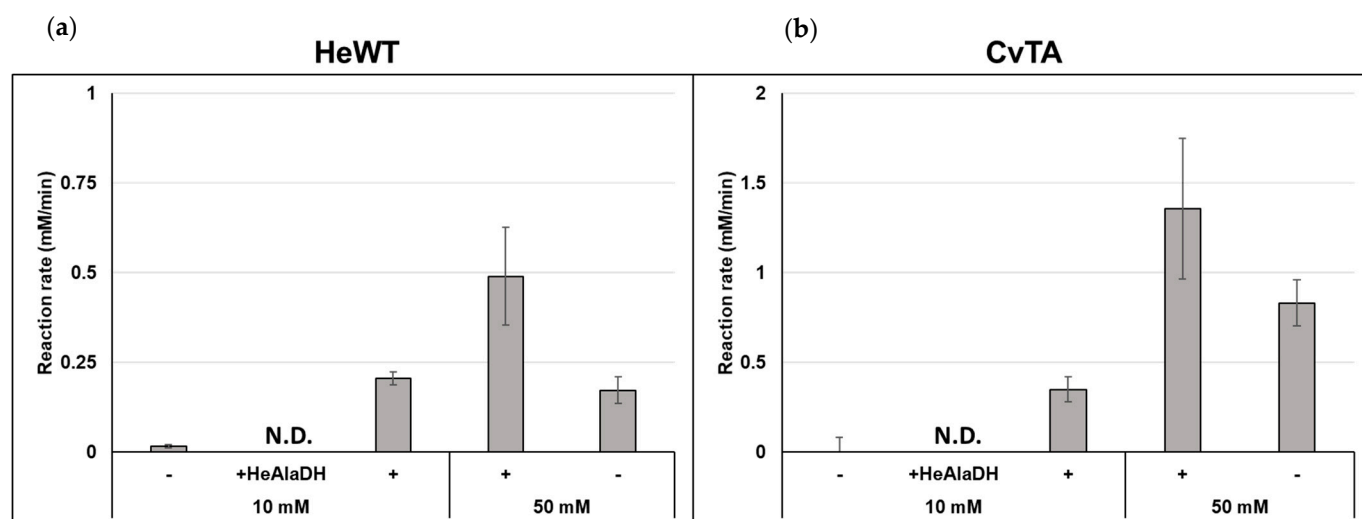


Figure 2. Reaction rate for the amination of vanillin. In the graphs on the left (a), the comparison between the transaminase alone (–), combined with the alanine dehydrogenase (+HeAlaDH) or the whole assembled system (+) are reported. On the right (b), the comparison of the reaction rate at 10 and 50 mM scale with different equivalents of the amino donor is depicted. Reactions were monitored for the first two hours. The reaction rate was estimated on the linear range. Reactions were performed with 2 mg/mL of TA, 1 mg/mL of HeAlaDH, 1 mg/mL CbFDH with 10 or 50 mM of vanillin, 10 or 50 mM of L-alanine, 100 mM ammonium formate, 0.1 eq. of NAD^+ in 100 mM phosphate buffer pH 8 with 10% DMSO. N.D. refers to not detected.

2.2. Enzyme Immobilisation

Once the system was characterised in a homogenous system, to increase the sustainability and specially, the reusability of the biocatalysts involved, immobilisation onto a solid support was tested. As HeWT, CvTA, and CbFDH had been previously immobilised [25,32,33], the attention was brought to HeAlaDH. Taking advantage of the presence of the His-tag in HeAlaDH, a previously described directed epoxy based covalent immobilisation technique was used [34].

Fixing the amount of immobilised enzyme to 0.5 mg/g, different supports for the covalent immobilisation of HeAlaDH in epoxide groups was attempted (Table S1). From the methacrylic supports, HFA403-S allowed up to a $32 \pm 2\%$ of recovered activity and epoxy agarose, with similar pore size and higher hydrophilicity, increasing the recovered activity to up to $42 \pm 1\%$. Despite the good recovered activities, the stability of the catalyst was poor, loosing up to 50% when reusing it 5 times. This is an unusual behaviour as many

immobilised enzymes acquire a much higher operational stability, however HeAlaDH is a hexamer and upon boiling the resin, it became obvious that not all the subunits were covalently immobilised (Figure S7). For multimeric enzymes, the loss of subunits has been seen before as one of the causes of poor reusability. To prevent this, several post-immobilisation coating techniques have proven to be effective [35–37]. In the case of HeAlaDH, glutaraldehyde (GA) seemed to have a negative effect, with almost a 15% decrease in the recovered activity, probably due to the capacity of this small molecule to permeate in the protein and potentially over-rigidifying the enzyme or even affecting the active site. On the other hand, bigger polymers such as polyethyleneimine (PEI) and dextran aldehyde seemed to have no impact on the recovered activity but allowed the creation of a biocatalyst with stable activity for at least 10 cycles.

Once the immobilisation of HeAlaDH was optimised, the creation of a redox-neutral recycling system was tested by co-immobilising CbFDH since both enzymes seemed to be necessary to increase the amination rate and co-immobilisation can enhance enzyme cooperativity [38]. In this case, HFA403-S, EC/HFA403-S, and Ep-Ag were selected due to their compatibility with both enzymes and the immobilisation was tested with sequential or simultaneous addition of the enzymes, followed by a PEI coating (Table S3).

Surprisingly, despite the lower recovered activities for CbFDH, the resin where the two enzymes were co-immobilised simultaneously outperformed the other preparations when tested in combination with soluble CvTA in the amination of vanillin (Figure 3). As seen before with the soluble enzyme, maintaining NADH available for the alanine dehydrogenase seems to be key to push the amination reaction.

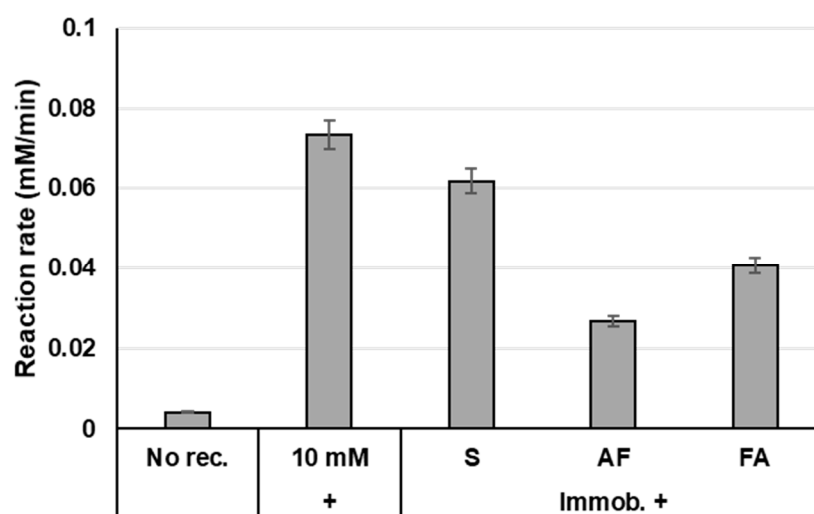


Figure 3. Reaction rate for the amination of vanillin. In all cases, the reaction consisted of 10 mM vanillin, 1 eq. of alanine, 250 mM ammonium formate, 1 mM NAD⁺, 0.1 mM PLP with 2 mg/mL of CvTA and 20 mg of the immobilised biocatalyst (S: simultaneous immobilisation; AF: HeAlaDH followed by CbFDH and FA: CbFDH followed by HeAlaDH).

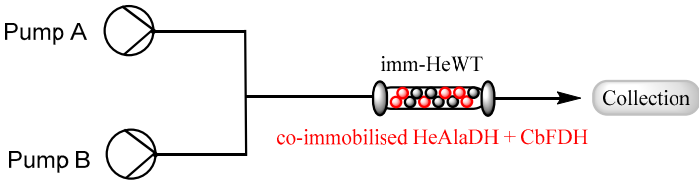
In conclusion, for the creation of a self-sufficient biocatalyst, the best performing preparation was the simultaneous immobilisation of HeAlaDH and CbFDH (1 mg/g support and 5 mg/g of support, respectively) with post-immobilisation coating with PEI to avoid HeAlaDH subunit loss during operation.

2.3. Scale Up: Continuous Flow and Reusability in Batch

In a step forward, the process intensification was attempted using the immobilised enzymes in a packed bed reactor using vanillin as substrate (Table 1). For the continuous flow, HeWT rather than CvTA was used due to its higher stability in flow [25,32]. In this case, 1 g of transaminase (the recovered activities are indicated in Table S4) was mixed with 2 g of co-immobilised HeAlaDH and CbFDH. With the methacrylic resins, although the

conversion reached up to 50%, the product remained trapped in the column. Conversion could be assessed by the remaining vanillin once the column was saturated. All efforts to solve this issue were unsuccessful, either because the use of a segmented flow with the addition of an immiscible organic solvent inactivated the cofactor recycling system, or because the final yield was worsened when increasing the ionic strength in the buffer, or with the addition of surfactants. In another attempt, the more hydrophilic resin (Ep-Ag) was tested to circumvent high substrate/product affinity for the support. In this case, recovery of the product was possible and while the transaminase alone only achieved 23% conversion independently of the retention time, the conversion doubled with the full system at longer retention time.

Table 1. Molar conversion of vanillin using a packed bed reactor. Pump A reservoir contained 20 mM vanillin in phosphate buffer pH8 with 20% (v/v) DMSO. Pump B reservoir contained 20 mM L-alanine, 200 mM ammonium formate, 2 mM NAD⁺ and 50 mM phosphate buffer pH8. The reaction consisted of 10 mM vanillin, 10 mM L-alanine, 100 mM ammonium formate, 1 mM NAD⁺ in 50 mM phosphate buffer pH8 with 10% DMSO. Conversions were assessed by HPLC.



Support	Retention Time	TA	TA + Rec
HFA403-S	10 min	<5%	22 ± 2
	20 min	<5%	50 ± 12
Ag-Ep	10 min	22 ± 5	29 ± 5
	20 min	23 ± 5	40 ± 5

Despite the improved results, these were far from what could be obtained in the batch reaction where in 2 h full conversion of both vanillin and cinnamaldehyde was achieved. Taking advantage of the better performance of the enzymes in their soluble form and to prevent the inactivation of HeFDH after immobilisation, another strategy was adopted. Very recently, it has been reported that the enclosure of a laccase in a dialysis bag retains the original activity of the enzyme and help its reusability, avoiding protein immobilisation [39].

To test the dialysis membrane enclosed system, the enzyme cocktail (1 mg/mL CvTA, 1 mg/mL HeAlaDH, and 2 mg/mL CbFDH) was placed inside a dialysis bag which was then put inside a reaction vessel containing up to 5 times the volume of the enzymatic mix. In this case, the reaction achieved completion over 24 h (Figure 4a) and, the enzymatic mix could be reused to some extent (Figure 4b) with both aromatic substrates, although conversion dropped to almost half for cinnamaldehyde already in the second cycle.

To overcome this low reusability, especially with cinnamaldehyde, the stabilising effect of different water-soluble polymers and small molecules was investigated. Previous studies found that polyols, such as polyethylene glycol (PEG) or small alcohols (glycerol and sugar moieties), can be used in the stabilisation of different biocatalysts [40]. On the other hand, polyamines such as polyethyleneimine (PEI) have also been related to increased stability of some proteins [41]. Based on these, 5 different molecules were tested: PEI, PEG, glycerol, and sucralose (Table 2).

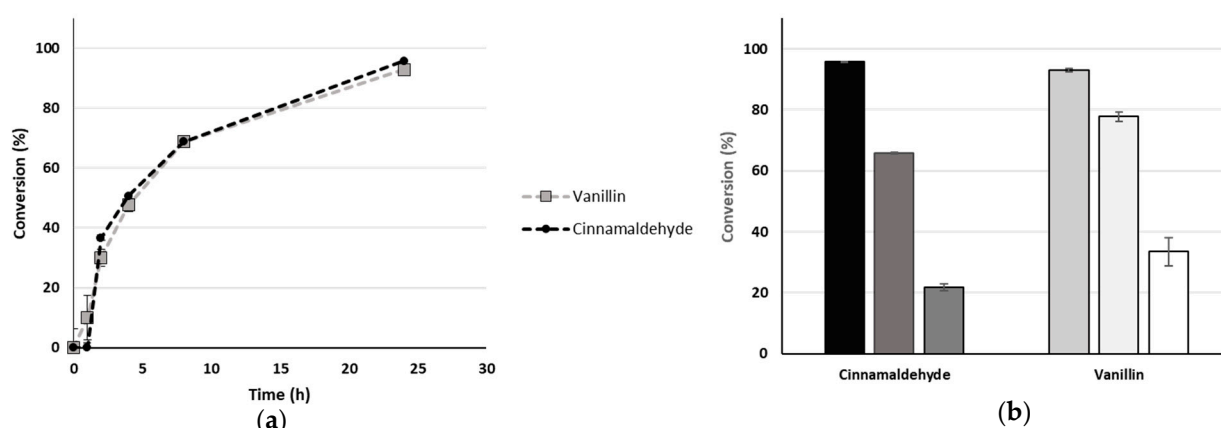


Figure 4. Conversion of cinnamaldehyde and vanillin to their corresponding amines in the dialysis system. (a) Conversion over time for the first cycle. (b) The reusability of the system is shown. The reaction mixture contained 10 mM of the corresponding aldehyde, 10 mM L-alanine, 250 mM ammonium formate, 1 mM NAD⁺, 1mM PLP and the enzymatic cocktail contained 1 mg/mL of CvTA, 1 mg/mL of HeAlaDH, and 2 mg/mL of CbFDH.

Table 2. Effect of different stabilising agents on the overall yield after 3 cycles of the dialysis assisted scale up. Reactions consisted of 4 mL of 10 mM cinnamaldehyde, 1 eq. of L-alanine, 250 mM ammonium formate, 1 mM NAD⁺, 0.1 mM PLP in 100 mM phosphate buffer pH8. The enzymatic mix was 1 mg/mL of CvTA, 1 mg/mL of HeAlaDH, and 2 mg/mL of HeFDH in phosphate buffer pH8 and a total volume of 0.5 mL. Each cycle was run for 24 h.

Additive	Concentration (mg/mL)	Accumulated Yield (%)	Relative Improvement
Control	-	52	1
PEI25	2.5	53	1
PEI60		48	0.9
PEI270		61	1.2
PEI750		51	1.0
PEG	25	51	1.0
	50	65	1.3
	100	57	1.1
Glycerol	100	54	1.1
	150	70	1.4
	200	72	1.4
Sucralose	10	55	1.1
	50	69	1.3
	100	57	1.1

From the different range of concentrations and additives tested, 50 mg/mL PEG, 15 mg/mL and 20% glycerol, and 50 mg/mL sucralose seemed to improve the reaction up to 1.4 times compared to the control, achieving around 70% accumulated conversion over the 3 cycles. These conditions were shown to stabilise the enzymes, at least for 24 h, with either no effect or increased activity after 24 h for all three enzymes. In the case of PEI, no real improvement could be seen in any of the cases, probably due to decreased stability of the transaminase and the formate dehydrogenase in those conditions. Interestingly though, HeAlaDH was hyperactivated in those conditions with almost 3 times more activity after 24 h with PEI of 60 kDa and higher mass (Figure S8).

3. Discussion

The production of amines, as building blocks for many nutraceutical molecules, is a major focus not only in research but also at the industrial level. Thus, the application of biocatalytic strategies to circumvent their instability in high concentration of amino donor is of special interest to access amines with unfavourable equilibria. Here, HeAlaDH proved to be a versatile enzyme due to its high resistance to organic solvents and different pH range to be applied in combination of a transaminase to push the equilibria further. In addition, here we observed that not only L-alanine recycling enhances the final yield, but the recycling of the cofactor is of key importance to avoid the competition between the transaminase and the alanine dehydrogenase for L-alanine as the substrate. Thus, in our case, the combination with a cofactor recycling enzyme is key to affect the transamination equilibria. This strategy has been used in two intensification processes: continuous flow and as dialysis enclosed biocatalyst. In the first approach however, the use of CbFDH as the cofactor recycling enzyme in its immobilized form lead to poorer performance, confirming previous reports which indicated its sensitivity to covalent immobilisation [33]. Although the remaining activity yielded decent conversion in our case, it poses a problem for its wider application as a cofactor recycling system. Other enzymes, such as glucose dehydrogenases [28], could be used for the recycling of the cofactor but complexity of the system is increased with the added substrate and product generated. To surpass this limitation, the use of other formate dehydrogenases, which pose the advantage of limited residues and excellent atom economy, capable to better withstand covalent immobilisation could greatly benefit the proposed system. On the other hand, when using methacrylate resins as support, their hydrophilicity had major effect on the product recovery, but the presence of the cascade allowed 2-fold higher concentrations in the same retention time. Nonetheless, the use of agarose as the support for immobilisation allowed continuous recovery of both product and substrate (with no retention on the column) with 40% conversions in 20 min retention time. Compared to the methacrylic resin, with almost the same conversion, the direct recovery of the product without any entrapment in the matrix poses a major advantage.

In the second approach, while the conversions matched with the ones with the free enzyme in the first cycle, subsequent reactions showed decreasing conversions which related to poor stability of the biocatalyst for long reaction times. To enhance their stability, medium engineering through the addition of stabilising agents was attempted. Following previous reports, polyethyleneimines and alcohol containing molecules were chosen for the tests [40–42]. While most of these additives are common in protein storage buffers (specially glycerol), we wanted to investigate if their effect would also translate in higher operational stability. It is also important to note that the use of polymers with high molecular weight (such as PEI or PEG) inside the dialysis membrane, which has a higher molecular weight cut off (MWCO), also prevents their leak to the reaction bulk. In the best conditions, a final yield of over 70% after 3 cycles could be obtained with glycerol at concentrations of at least 150 mg/mL, followed by PEG and sucralose at 50 mg/mL which also had a beneficial effect. These results, in combination with the stability assays performed with the same additives, clearly indicate that for this enzymatic cocktail, alcohol containing molecules are the best choice as they have a beneficial effect for all the enzymes involved. PEI on the other hand, has a positive effect for HeAlaDH but results in reduced stability for both the transaminase and the CbFDH. These results indicate that the choice of stabilising agents highly depends on the enzymatic system used. It is important to note here, that while the substrate concentration is the same as in the free enzyme biotransformations in each reaction, bigger reaction volumes can be used without scaling up the amount of enzyme, which multiplies the total turnover number of the enzyme.

4. Materials and Methods

4.1. Materials, Strains, Vectors, and Culture Conditions

All chemical reagents, unless stated otherwise, were purchased as analytical grade from Sigma-Aldrich, Gillingham, U.K., Acros Organics or Thermo Fischer Scientific, Lough-

borough, U.K. NAD⁺ was purchased from Apollo Scientific Ltd., Stockport, U.K. Sepabeads and Relyzime supports for enzyme immobilisation were kindly provided by Residindion S.L. Agarose 6 BCL was purchased from Agarose Bead Technologies (ABT), Madrid, Spain.

4.2. Protein Expression and Purification

For HeWT and CvTA, one single colony was inoculated directly in the flask with either 50 or 300 mL of ZYP-5052 media supplemented with the ampicillin (100 µg/mL) and allowed to grow for 20 h at 37 °C. For the other two proteins (HeAlaDH and CbFDH), an overnight pre-inoculum of at least 10 mL was prepared the day before and 1/100 of the final volume added to either 50 or 300 mL. HeAlaDH was expressed in Terrific Broth media supplemented with ampicillin (100 µg/mL) and CbFDH in LB media supplemented with kanamycin (30 µg/mL). When the OD₆₀₀ of these last two reached 0.6–0.8, protein expression was induced with the addition of 1 mM IPTG and the cultures were allowed to grow for 20 h at 30 °C. After that, cell cultures were harvested at 4000 rpm for 20 min at 4 °C in the appropriate centrifuge tubes and the cells separated from the medium. The supernatant was carefully removed, and the cells resuspended in a minimum of 2 mL of loading buffer/g of pellet. The cell lysis was performed in ice using the sonicator in pulse mode (5 s on, 10 s off) for a minimum of 5 min. The lysate was then centrifuged at 14,500 rpm, for a minimum of 45 min at 4 °C. The collected supernatant was filtered with 0.45 µm Millex PVDF filters before loading onto the Ni²⁺ preloaded columns. The affinity chromatography was performed using an AKTA Start system with the appropriate column. The filtered crude extract was loaded and left washing until the non-specific proteins were completely eluted. After that, an isocratic wash step with only 5–10% of elution buffer was performed to elute the non-specific proteins still bound onto the column. Finally, 100% of elution buffer was passed through the column and protein elution monitored by UV. Fractions were collected and those containing the desired protein pooled and placed into dialysis tubing. The protein samples were dialysed at least for 20 h replacing the buffer at least 2 times at 4 °C. All proteins were dialysed against 50 mM phosphate buffer pH8 and 0.1 mM of PLP was added for HeWT. The pure proteins were stored at 4 °C. Protein quantification was performed using a Take3 plate in an EPOCH2 by measuring the absorbance at 280 nm. The molar extinction coefficient and molecular mass are: 24,500 M^{−1} cm^{−1} and 42 KDa for HeAlaDH, 51,402 M^{−1} cm^{−1} and 42 KDa for CbFDH, 62,840 M^{−1} cm^{−1} and 54.4 KDa for HeWT, and 81,735 M^{−1} cm^{−1} and 55.2 kDa for CvTA.

4.3. Enzymatic Assay

For the soluble enzyme, the activity was measured in the oxidative deamination with 40 mM alanine and 1 mM NAD⁺ with the appropriate amount of enzyme in 100 mM glycine buffer pH 10 or pH 8. For the reductive amination, 2.5 mM pyruvate, 250 mM NH₄⁺, and 0.5 mM of NADH with an appropriate amount of enzyme in 100 mM phosphate buffer pH8. For CbFDH, the activity assay was performed with 100 mM of ammonium formate and 1 mM NAD⁺ in phosphate buffer pH8. The formation or depletion of the cofactor was followed by measuring the absorbance at 340 nm ($\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) and for HeWT, the activity assay was performed with 2.5 mM pyruvate, 2.5 mM SMBA, and 0.1 mM PLP in phosphate buffer pH8 following the formation of acetophenone at 245 nm ($\epsilon = 12,000 \text{ M}^{-1} \text{ cm}^{-1}$).

A unit defined as the µmols of either product formed or substrate depleted per minute.

For the immobilised enzyme, between 20 and 50 mg of resin were weighted, and the reaction performed in a total volume of 5 mL measuring the desired absorbance every two minutes for a total time of 10 min with the same reaction conditions as stated before. The expressed activity (U/g) and recovered activity (%) were calculated as follows:

$$\text{Expressed activity (\%)} = \frac{\text{UA/min} \times \text{volume of reaction (mL)}}{\epsilon (\text{mM}^{-1} \text{cm}^{-1}) \times \text{Pathlength (cm)} \times \text{g of imm.biocatalyst}}$$

$$\text{Recovered activity(\%)} = \frac{\text{U/mg (immobilised enzyme)}}{\text{U/mg of enzyme (free)}} \times 100$$

4.4. SDS-PAGE

SDS-PAGE assay was performed following the original procedure [43]. The running gel (12%: 1.95 mL of Tris HCl 1.5 M pH 8.8, 2.25 mL acrylamide 40%, 3.125 mL of dH₂O, 75 µL SDS 10%, 75 µL ammonium persulfate 10% (*w/v*), and 7.5 µL TEMED) was prepared and loaded in between the two glass pieces, adding a few drops of isopropanol on top to avoid the formation of a meniscus. After the gel was polymerised, the stacking gel was prepared and loaded (0.25 mL 1 M Tris pH 6.8, 0.33 mL acrylamide 40%, 1.4 mL dH₂O, 20 µL SDS 10%, 20 µL ammonium persulfate 10% (*w/v*), and 3 µL TEMED). Before loading, the samples were heated at 90 °C for at least 5 min after mixed with an equal volume of the 2× loading buffer (0.18 M Tris-HCl buffer pH6, 3.8 mM β-mercaptoethanol, 7.2% (*w/v*) SDS, 36% (*w/v*) glycerol, and 0.36 (*w/v*) bromophenol blue). The assay was run at 30 mA, 300 V for 70 min. The protein marker, unstained protein standard, broad range (10–200 kDa) was loaded as a comparison. The gel was then removed from the mould and either stained with Coomassie blue staining solution (2% Coomassie brilliant blue R-250 in aqueous solution 50% methanol and 10% acetic acid) for 15–30 min following destaining with the destaining solution (aqueous solution 7.5% methanol and 10% acetic acid) overnight or with Instant Blue (Expedeon®) solution overnight.

4.5. Batch Reactions

Batch biotransformations with free enzyme were performed with the desired concentration of substrate, 1–2 mg/mL of enzyme in phosphate buffer pH8. Samples were withdrawn at different times and the reaction was quenched by adding 450 µL of HCl 0.2% and 450 µL of acetonitrile. The samples were analysed by HPLC (Dionex UltiMate 3000 (Thermo Fisher, Loughborough, UK), Waters X-Bridge C18 (Waters, Elstree, UK) (3.5 µm, 2.1 × 100 mm), 0.8 mL/min, measuring at 210, 250, and 265 nm) to assess the conversion.

4.6. Immobilisation of HeAlaDH into Epoxy Functionalised Supports

The support was prepared following previous indicated protocol [27]. For the protein immobilisation, 2 mL/g resin with the desired amount of protein in 50 mM phosphate buffer pH8 were mixed at RT for 4 or 16 h, when no further decrease in the activity could be detected in the supernatant. The immobilisation yield was calculated as the percentage of protein remaining in the supernatant after incubation with respect to the offered protein.

4.7. Preparation of Dextran-Aldehyde

To obtain the 50% oxidised dextran-aldehyde, 10 g of dextran (35–45 KDa) were dissolved in 100 mL water and mixed with 1 g of periodate stirring at room temperature for 2 h. After, it was dialysed against 50 times the volume of distilled water 4 times.

4.8. Post Immobilisation Coating of Immobilised HeAlaDH

After the immobilisation, 10 volumes of 5 mg/mL PEI in 100 mM carbonate buffer pH10 were mixed with the resin for 1 h at RT. After that, the resin was filtered and washed thoroughly with water and 50 mM phosphate buffer pH8 and stored at 4 °C in the 50 mM phosphate buffer pH8. For glutaraldehyde, 10 volumes of 5 mg/mL glutaraldehyde in 50 mM phosphate buffer pH8 were mixed with the resin for 1 h at RT. Sequentially, 1 mg/mL of sodium borohydride was added, and the mix shaken gently for 20 min. After that, the resin was filtered and washed thoroughly with water and 50 mM phosphate buffer pH8 and stored at 4 °C in the 50 mM phosphate buffer pH8. The coating method was modified from previously reported protocols. To conclude, for dextran coating, 100 mg of immobilised resin were resuspended in 500 µL of 50 mM phosphate buffer pH 8 along with the appropriate amount of dextran poly-aldehyde and left stirring O/N. After that, 1 mg/mL of sodium borohydride was added along with 50 µL of 900 mM bicarbonate

buffer pH10 and the mix was left stirring at 4 °C for 25 min before it the biocatalyst was washed thoroughly with water and stored in 50 mM phosphate buffer pH 8.

4.9. Co-Immobilisation of HeAlaDH and CbFDH

To the previously activated support, either of 5 mg/g of resin of CbFDH or 1 mg/g of resin of HeAlaDH were added and left mixing room temperature. After that, the percentage of immobilised protein was assessed with both the remaining activity and the protein concentration in the supernatant. Then, 1 mg/g of resin of HeAlaDH or 5 mg/g of resin of CbFDH were added and left mixing with the support at room temperature. For HeAlaDH, the mixing time was 4 h while for CbFDH, O/N incubation was needed to achieve the maximum immobilisation yield. Desorption and blocking were performed as detailed before for HeAlaDH with PEI.

4.10. Flow Reactions

Continuous flow biotransformations were performed using a R2 + /R4 flow reactor commercially available from Vapourtec® equipped with an Omnifit® glass column (6.6 mm i.d. × 100 mm length) filled with an appropriate volume of immobilised enzyme. When needed, two solutions or solvents were mixed using a T tube before entrance to the column. The flow rate was varied and optimised for each reaction. The exiting flow stream was collected, and the results analysed by HPLC. The samples were analysed by HPLC (Dionex UltiMate 3000, Waters X-Bridge C18 (3.5 µm, 2.1 × 100 mm), 0.8 mL/min, measuring at 210, 250, and 265 nm) to assess the conversion.

4.11. Dialysis Assisted Reaction

A mix of enzymes at the desired concentration were added inside a dialysis membrane with a cut-off (MWCO) of 12 KDa (D9527-100FT from Sigma Aldrich, St. Louis, MO, USA). The membrane secured in both sides and submerged into a glass vial containing 5 times the volume of enzyme cocktail of reaction mixture. The glass vial was left stirring at 37 °C for at least 24 h. At the desired timepoints, samples were taken from the reaction bulk and analysed by HPLC.

4.12. HPLC Analysis

Typically, for compounds with detectable chromophores, samples were appropriately diluted in a solution of 1:1 (*v/v*) 0.1% HCl and 450 µL of MeCN to stop the enzymatic reaction. These samples were then analysed by HPLC (Dionex UltiMate 3000, Waters X-Bridge C18 (3.5 µm, 2.1 × 100 mm), measuring at 210, 250, and 265 nm to assess the conversion using a gradient method from 5:95 to 95:5 (H₂O:MeCN 0.1% TFA) over 4 min with a flow rate of 0.8 mL/min.

For compounds with an amine functionality with no detectable fluorophore, FMOC derivatisation was used to enable their detection. Typically, 100 µL of a maximum of 10 mM of the desired compound were mixed with 200 µL of 100 mM borate buffer pH 9. To the mix, 400 µL of 15 mM FMOC diluted in MeCN were added. The sample was properly mixed and left for at least 10 min before mixing 200 µL of it with 400 µL of MeCN and 400 µL of 0.1% HCl. Samples were run using a gradient method from 40:60 to 95:5 (H₂O:MeCN 0.1% TFA) over 4 min with a flow rate of 0.8 mL/min.

The retention times for the different chemicals were: 3.09 min for vanillin, 4.14 min for cinnamaldehyde, 3.12 min for cinnamyl amine, 1.58mins for alanine-FMOC, and 4.13 min for cyclohexylamine-FMOC.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/catal11040520/s1>. Figure S1. SDS-page analysis of HeAlaDH expression, Figure S2. Kinetic characterisation of HeAlaDH in both the oxidative deamination and the reductive amination direction, Figure S3. Cosolvent effect on the stability of HeAlaDH, Figure S4. pH effect on the stability of HeAlaDH, Figure S5. Amination of vanillin at the 10 mM scale with different equivalents of alanine and the presence (+) or absence (-) of the cascade for the amino donor recycling, Figure S6. Amination

of vanillin at the 10- and 50-mM scale with 1 equivalent of alanine, Figure S7. A. SDS-PAGE analysis of the immobilised biocatalysts, Figure S8. Stability of the different biocatalysts in the presence of the different additives, Table S1: Results of the immobilisation of HeAlaDH in various supports, Table S3: Co-immobilization of HeAlaDH (1 mg/g) and CbFDH (5 mg/g) in the three different tested resins, Table S4. Recovered activities and specific activity of the biocatalysts of the three different enzymes.

Author Contributions: Conceptualisation, F.P.; methodology, F.P. and D.R.P.; experimental Z.N. and D.R.P.; writing—original draft preparation, D.R.P.; writing—review and editing, F.P.; All authors have read and agreed to the published version of the manuscript.

Funding: The authors wish to acknowledge the Biotechnology and Biological Research Council [grant number BB/P002536/1] and H2020 ERA-CoBioTech programme (project ID: 61 HOMBIOCAT) also funded through the Biotechnology and Biological Sciences Research Council [grant number BB/R021287/1].

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, F.P., upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Gröger, H. Biocatalytic concepts for synthesizing amine bulk chemicals: Recent approaches towards linear and cyclic aliphatic primary amines and ω -substituted derivatives thereof. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 83–95. [\[CrossRef\]](#)
- Pelckmans, M.; Renders, T.; Van De Vyver, S.; Sels, B.F. Bio-based amines through sustainable heterogeneous catalysis. *Green Chem.* **2017**, *19*, 5303–5331. [\[CrossRef\]](#)
- Kelly, S.A.; Pohle, S.; Wharry, S.; Mix, S.; Allen, C.C.R.; Moody, T.S.; Gilmore, B.F. Application of ω -Transaminases in the Pharmaceutical Industry. *Chem. Rev.* **2018**, *118*, 349–367. [\[CrossRef\]](#) [\[PubMed\]](#)
- Galman, J.L.; Slabu, I.; Weise, N.J.; Iglesias, C.; Parmeggiani, F.; Lloyd, R.C.; Turner, N.J. Biocatalytic transamination with near-stoichiometric inexpensive amine donors mediated by bifunctional mono- and di-amine transaminases. *Green Chem.* **2017**, *19*, 361–366. [\[CrossRef\]](#)
- Cassimjee, K.E.; Manta, B.; Himo, F. A quantum chemical study of the ω -transaminase reaction mechanism. *Org. Biomol. Chem.* **2015**, *13*, 8453–8464. [\[CrossRef\]](#)
- Savile, C.K.; Janey, J.M.; Mundorff, E.C.; Moore, J.C.; Tam, S.; Jarvis, W.R.; Colbeck, J.C.; Krebber, A.; Fleitz, F.J.; Brands, J.; et al. Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture. *Science* **2010**, *329*, 305–309. [\[CrossRef\]](#) [\[PubMed\]](#)
- Martin, A.R.; Shonnard, D.; Pannuri, S.; Kamat, S. Characterization of free and immobilized (S)-aminotransferase for acetophenone production. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 843–851. [\[CrossRef\]](#)
- Guo, F.; Berglund, P. Transaminase biocatalysis: Optimization and application. *Green Chem.* **2017**, *19*, 333–360. [\[CrossRef\]](#)
- Börner, T.; Rämisch, S.; Bartsch, S.; Vogel, A.; Adlercreutz, P.; Grey, C. Three in One: Temperature, Solvent and Catalytic Stability by Engineering the Cofactor-Binding Element of Amine Transaminase. *ChemBioChem* **2017**, *18*, 1482–1486. [\[CrossRef\]](#) [\[PubMed\]](#)
- Roura Padrosa, D.; Alaux, R.; Smith, P.; Dreveny, I.; López-Gallego, F.; Paradisi, F. Enhancing PLP-Binding Capacity of Class-III ω -Transaminase by Single Residue Substitution. *Front. Bioeng. Biotechnol.* **2019**, *7*, 282. [\[CrossRef\]](#)
- Börner, T.; Rämisch, S.; Reddem, E.R.; Bartsch, S.; Vogel, A.; Thunnissen, A.M.W.H.; Adlercreutz, P.; Grey, C. Explaining Operational Instability of Amine Transaminases: Substrate-Induced Inactivation Mechanism and Influence of Quaternary Structure on Enzyme-Cofactor Intermediate Stability. *ACS Catal.* **2017**, *7*, 1259–1269. [\[CrossRef\]](#)
- Kelefiotis-Stratidakis, P.; Tyrikos-Ergas, T.; Pavlidis, I.V. The challenge of using isopropylamine as an amine donor in transaminase catalysed reactions. *Org. Biomol. Chem.* **2019**, *17*, 1634–1642. [\[CrossRef\]](#)
- Huisman, G.W.; Collier, S.J. On the development of new biocatalytic processes for practical pharmaceutical synthesis. *Curr. Opin. Chem. Biol.* **2013**, *17*, 284–292. [\[CrossRef\]](#)
- Leipold, L.; Dobrijevic, D.; Jeffries, J.W.E.; Bawn, M.; Moody, T.S.; Ward, J.M.; Hailes, H.C. The identification and use of robust transaminases from a domestic drain metagenome. *Green Chem.* **2019**, *21*, 75–86. [\[CrossRef\]](#)
- Green, A.P.; Turner, N.J.; Reilly, E.O. Chiral Amine Synthesis Using ω -Transaminases: An Amine Donor that Displaces Equilibria and Enables High-Throughput Screening. *Angewandte* **2014**, 10714–10717. [\[CrossRef\]](#) [\[PubMed\]](#)
- Baud, D.; Ladkau, N.; Moody, T.S.; Ward, J.M.; Hailes, H.C. A rapid, sensitive colorimetric assay for the high-throughput screening of transaminases in liquid or solid-phase. *Chem. Commun.* **2015**, *51*, 17225–17228. [\[CrossRef\]](#) [\[PubMed\]](#)
- Slabu, I.; Galman, J.L.; Weise, N.J.; Lloyd, R.C.; Turner, N.J. Putrescine Transaminases for the Synthesis of Saturated Nitrogen Heterocycles from Polyamines. *ChemCatChem* **2016**, *8*, 1038–1042. [\[CrossRef\]](#)
- Martínez-Montero, L.; Gotor, V.; Gotor-Fernández, V.; Lavandera, I. Stereoselective amination of racemic sec-alcohols through sequential application of laccases and transaminases. *Green Chem.* **2017**, *19*, 474–480. [\[CrossRef\]](#)
- Koszelewski, D.; Lavandera, I.; Clay, D.; Guebitz, G.M.; Rozzell, D.; Kroutil, W. Formal asymmetric biocatalytic reductive amination. *Angew. Chem. Int. Ed.* **2008**, *47*, 9337–9340. [\[CrossRef\]](#) [\[PubMed\]](#)

20. Richter, N.; Farnberger, J.E.; Pressnitz, D.; Lechner, H.; Zepeck, F.; Kroutil, W. A system for ω -transaminase mediated (R)-amination using L-alanine as an amine donor. *Green Chem.* **2015**, *17*, 2952–2958. [\[CrossRef\]](#)
21. Klatte, S.; Wendisch, V.F. Role of L-alanine for redox self-sufficient amination of alcohols. *Microb. Cell Fact.* **2015**, *14*, 1–10. [\[CrossRef\]](#)
22. Koszelewski, D.; Lavandera, I.; Clay, D.; Rozzell, D.; Kroutil, W. Asymmetric synthesis of optically pure pharmacologically relevant amines employing ω -transaminases. *Adv. Synth. Catal.* **2008**, *350*, 2761–2766. [\[CrossRef\]](#)
23. Contente, M.L.; Dall'Oglio, F.; Tamborini, L.; Molinari, F.; Paradisi, F. Highly Efficient Oxidation of Amines to Aldehydes with Flow-based Biocatalysis. *ChemCatChem* **2017**, *9*, 3843–3848. [\[CrossRef\]](#)
24. Abaházi, E.; Sátorhelyi, P.; Erdélyi, B.; Vértessy, B.G.; Land, H.; Paizs, C.; Berglund, P.; Poppe, L. Covalently immobilized Trp60Cys mutant of Ω -transaminase from *Chromobacterium violaceum* for kinetic resolution of racemic amines in batch and continuous-flow modes. *Biochem. Eng. J.* **2018**, *132*, 270–278. [\[CrossRef\]](#)
25. Planchestainer, M.; Contente, M.L.; Cassidy, J.; Molinari, F.; Tamborini, L.; Paradisi, F. Continuous flow biocatalysis: Production and in-line purification of amines by immobilised transaminase from *Halomonas elongata*. *Green Chem.* **2017**, *19*, 372–375. [\[CrossRef\]](#)
26. Truppo, M.D.; Strotman, H.; Hughes, G. Development of an Immobilized Transaminase Capable of Operating in Organic Solvent. *ChemCatChem* **2012**, *4*, 1071–1074. [\[CrossRef\]](#)
27. Agustian, J.; Kamaruddin, A.H.; Bhatia, S. Enzymatic membrane reactors: The determining factors in two separate phase operations. *J. Chem. Technol. Biotechnol.* **2011**, *86*, 1032–1048. [\[CrossRef\]](#)
28. Possebom, G.; Nyari, N.L.D.; Zeni, J.; Steffens, J.; Rigo, E.; di Luccio, M. Esterification of fatty acids by *Penicillium crustosum* lipase in a membrane reactor. *J. Sci. Food Agric.* **2014**, *94*, 2905–2911. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Kaulmann, U.; Smithies, K.; Smith, M.E.B.; Hales, H.C.; Ward, J.M. Substrate spectrum of ω -transaminase from *Chromobacterium violaceum* DSM30191 and its potential for biocatalysis. *Enzym. Microb. Technol.* **2007**, *41*, 628–637. [\[CrossRef\]](#)
30. Cerioli, L.; Planchestainer, M.; Cassidy, J.; Tessaro, D.; Paradisi, F. Characterization of a novel amine transaminase from *Halomonas elongata*. *J. Mol. Catal. B Enzym.* **2015**, *120*, 141–150. [\[CrossRef\]](#)
31. Schütte, H.; Flossdorf, J.; Sahm, H.; Kula, M.-R. Purification and Properties of Formaldehyde Dehydrogenase and Formate Dehydrogenase from *Candida boidinii*. *Eur. J. Biochem.* **1976**, *62*, 151–160. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Benítez-Mateos, A.I.; Contente, M.L.; Velasco-Lozano, S.; Paradisi, F.; López-Gallego, F. Self-Sufficient Flow-Biocatalysis by Coimmobilization of Pyridoxal 5'-Phosphate and ω -Transaminases onto Porous Carriers. *ACS Sustain. Chem. Eng.* **2018**, *6*, 13151–13159. [\[CrossRef\]](#)
33. Bolivar, J.M.; Wilson, L.; Ferrarotti, S.A.; Fernandez-Lafuente, R.; Guisan, J.M.; Mateo, C. Evaluation of different immobilization strategies to prepare an industrial biocatalyst of formate dehydrogenase from *Candida boidinii*. *Enzym. Microb. Technol.* **2007**, *40*, 540–546. [\[CrossRef\]](#)
34. Mateo, C.; Grazú, V.; Pessela, B.C.C.; Montes, T.; Palomo, J.M.; Torres, R.; López-Gallego, F.; Fernández-Lafuente, R.; Guisán, J.M. Advances in the design of new epoxy supports for enzyme immobilization–stabilization. *Biochem. Soc. Trans.* **2008**, *35*, 1593–1601. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Bolivar, J.M.; Rocha-Martín, J.; Mateo, C.; Guisan, J.M. Stabilization of a highly active but unstable alcohol dehydrogenase from yeast using immobilization and post-immobilization techniques. *Process Biochem.* **2012**, *47*, 679–686. [\[CrossRef\]](#)
36. Lopez-Gallego, F.; Betancor, L.; Hidalgo, A.; Dellamora-Ortiz, G.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J.M. Stabilization of different alcohol oxidases via immobilization and post immobilization techniques. *Enzym. Microb. Technol.* **2007**, *40*, 278–284. [\[CrossRef\]](#)
37. Trobo-Maseda, L.; Orrego, A.H.; Moreno-Pérez, S.; Fernández-Lorente, G.; Guisan, J.M.; Rocha-Martín, J. Stabilization of multimeric sucrose synthase from *Acidithiobacillus caldus* via immobilization and post-immobilization techniques for synthesis of UDP-glucose. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 773–787. [\[CrossRef\]](#)
38. Arana-Peña, S.; Carballares, D.; Morellon-Sterling, R.; Berenguer-Murcia, Á.; Alcántara, A.R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Enzyme co-immobilization: Always the biocatalyst designers' choice ... or not? *Biotechnol. Adv.* **2020**, 107584. [\[CrossRef\]](#)
39. Zhang, J.; Li, F.; Wang, R.; Tan, X.; Hagedoorn, P.-L. Dialysis membrane enclosed laccase catalysis combines a controlled conversion rate and recyclability without enzyme immobilization. *AMB Express* **2020**, *10*, 19. [\[CrossRef\]](#)
40. Bommaris, A.S.; Paye, M.F. Stabilizing biocatalysts. *Chem. Soc. Rev.* **2013**, *42*, 6534. [\[CrossRef\]](#)
41. Andersson, M.M.; Hatti-Kaul, R. Protein stabilising effect of polyethyleneimine. *J. Biotechnol.* **1999**, *72*, 21–31. [\[CrossRef\]](#)
42. Simpson, R.J. Stabilization of proteins for storage. *Cold Spring Harb. Protoc.* **2010**, *5*, pdb.top79. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685. [\[CrossRef\]](#) [\[PubMed\]](#)