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Biosynthesis of Furfurylamines in Batch and Continuous Flow by Immobilized Amine Transaminases

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Abstract: Building blocks with amine functionality are crucial in the chemical industry. Biocatalytic syntheses and chemicals derived from renewable resources are increasingly desired to achieve sustainable production of these amines. As a result, renewable materials such as furfurals, especially furfurylamines like 5-(hydroxymethyl)furfurylamine (HMFA) and 2,5-di(aminomethyl)furan (DAF), are gaining increasing attention. In this study, we identified four different amine transaminases (ATAs) that catalyze the reductive amination of 5-(hydroxymethyl)furfural (HMF) and 2,5-diformylfuran (DFF). We successfully immobilized these ATAs on glutaraldehyde-functionalized amine beads using multiple binding and on amine beads by site-selective binding of the unique Cα-formylglycine within an aldehyde tag. All immobilized ATAs were efficiently reused in five repetitive cycles of reductive amination of HMF with alanine as co-substrate, while the ATA from *Silicibacter pomeroyi* (ATA-Spo) also exhibited high stability for reuse when isopropylamine was used as an amine donor. Additionally, immobilized ATA-Spo yielded high conversion in the batch syntheses of HMFA and DAF using alanine (87% and 87%, respectively) or isopropylamine (99% and 98%, respectively) as amine donors. We further demonstrated that ATA-Spo was effective for the reductive amination of HMF with alanine or isopropylamine in continuous-flow catalysis with high conversion up to 12 days (48% and 41%, respectively).

Keywords: amine transaminase; biocatalysis; DFF; flow synthesis; HMF; immobilization; isopropylamine; reuse stability

Citation: Heinks, T.; Merz, L.M.; Liedtke, J.; Höhne, M.; van Langen, L.M.; Bornscheuer, U.T.; Fischer von Mollard, G.; Berglund, P. Biosynthesis of Furfurylamines in Batch and Continuous Flow by Immobilized Amine Transaminases. *Catalysts* **2023**, *13*, 875. https://doi.org/10.3390/catal13050875

Academic Editor: Rong Shi

Received: 31 March 2023
Revised: 29 April 2023
Accepted: 9 May 2023
Published: 11 May 2023

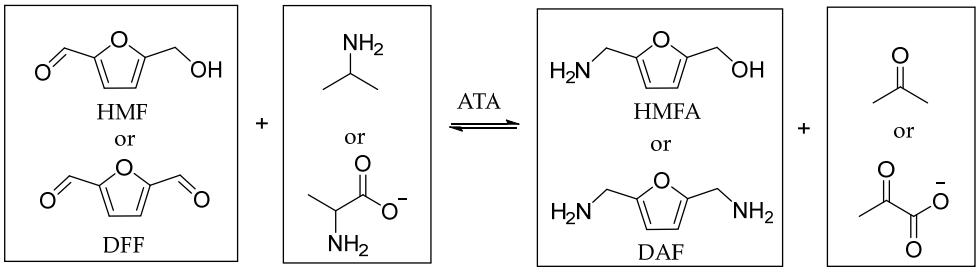
1. Introduction

Amines, particularly chiral amines, are crucial building blocks in the pharmaceutical industry, with approx. 40–45% of drug candidates containing an amine function [1]. Additionally, compounds with amine functionality also have applications in dyes, polymers, and agrochemicals [2,3]. While chiral amines are still commonly produced chemically, e.g., by reductive amination of ketones (e.g., [4]), biocatalysis offers a more sustainable alternative for their production in line with the principles of Green Chemistry [5,6]. Amine transaminases (ATAs), next to amine oxidases [7,8], imine reductases [9,10], or lipases [11,12], are attracting growing interest for large-scale synthesis of (chiral) amines because they offer high enantioselectivity, internal cofactor regeneration, and can accept a variety of different substrates. Amine transaminases transfer the amino-group of an amino

donor to an amine acceptor using the cofactor pyridoxal-5′-phopsphate (PLP), regardless of the position of the functional group, and unlike α-amino acid transaminases, they can operate regardless of the presence of a carboxyl group [13–16].

The sustainability of the enzymatic process can be further increased by using renewable materials. As such, furfurals have gained increasing interest in recent years as they can be produced from renewable raw materials and serve as interesting building blocks for various chemicals as well as bio-based fuels [17–25]. As a result, furfurals are among the top 30 value-added chemicals from biomass, according to the US Department of Energy [6]. In particular, furfurylamines are relevant intermediates in the production of pharmaceuticals, including diuretics [26] and antiseptics [27], or as intermediates for fungicides [28], herbicides, and pesticides [29]. Two furfurylamines of interest are 5-(hydroxymethyl)furfurylamine (HMFA) and 2,5-di(aminomethyl)furan (DAF), which are generated from 5-(hydroxymethyl)furfural (HMF) and 2,5-diformylfuran (DFF), respectively. Both HMF and DFF can be produced from renewable resources. HMF occurs naturally in products such as honey, toasted bread, or coffee [20,30–32], and it can be synthesized from fructose, isomerized from glucose [33], or retrieved sustainably from food waste biomass [34,35]. DFF, which can be used as a crosslinker for enzyme immobilization [36], can be produced from fructose or glucose [37]. Both HMFA and DAF are relevant building blocks in the pharmaceutical industry. Moreover, DAF, with its two terminal amino groups, is an interesting compound for the polymer industry, as it can be used as a raw material for polyamides [3], polyureas [38], and polyurethanes [39]. To date, only a few publications have focused on the enzymatic amination of HMF and DFF [17,24,40,41], while most studies aim at the chemical synthesis of HMFA and DAF (e.g., [18,21,42,43]). For biocatalysis to become an attractive and competitive alternative to conventional chemical synthesis, a stable and easy-to-use enzyme is necessary [44]. Several research efforts have been made to increase the stability of enzymes, for example by genetic mutation or adapting the reaction environment [45–50]. One popular strategy is immobilization of the enzymes, which has shown promising results in increasing the stability of different transaminases under various conditions so far [51–53]. Moreover, immobilization of the catalyst allows for easy removal from the reaction solution, reuse of the enzyme for several reaction cycles or in enzyme cascades, and, in some cases, modification of the stability and activity [35–47,51,52,54–64]. However, as each enzyme exhibits a different behavior when immobilized, the immobilization process needs to be newly established for each biocatalyst [48]. In general, immobilization can be achieved through a vast variety of techniques and with various materials and is mainly divided into two groups: carrier-dependent or carrier-free immobilized enzymes [55]. While carrier-free immobilization is possible through crosslinking of the enzymes themselves, carrier-bound immobilization requires a carrier such as a bead, fiber, capsule, film, or membrane [55]. The enzyme is either adsorbed on the carrier or covalently bound to it using a crosslinker such as glutaraldehyde [65], bisepoxide [66], or DFF [36]. Many carrier materials have been successfully applied for the immobilization of transaminases, including silica [56], chitosan [60,67], resins [51,61,66], epoxy beads [57], cellulose [68], PVA-Fe3O4 nanoparticles [69], MnO_2 nanorods [70], controlled porosity glass [71,72], or lignin [73].

Another major benefit of using immobilized enzymes for biocatalysis is the possibility of using the catalyst in a continuously operated flow reactor. Compared to batch catalysis, catalysis in flow offers benefits such as reduced product inhibition, continuous product removal, or improved heat and mass transfer [74,75]. Immobilized transaminases have been used in continuous flow reactions for the kinetic resolution of amines [56,66,72,76] or the synthesis of amines [68,73,77–85]. In this study, different ATAs were used for the reductive amination of HMF and DFF to HMFA and DAF, respectively, using alanine and isopropylamine as amine donors (Scheme 1). These ATAs have been immobilized using two different strategies (i.e., site-selective and multiple binding to amine and glutaraldehyde-functionalized amine beads, respectively) and analyzed in batch synthesis of HMFA and DAF and in continuous-flow synthesis of HMFA on a preparative scale.



Scheme 1. Reaction scheme for the transaminase-catalyzed amination of 5-(hydroxymethyl)furfural (HMF) and 2,5-diformylfuran (DFF) using isopropylamine or alanine as amine donors, yielding 5-(hydroxymethyl)furfurylamine (HMFA) or 2,5-di(aminomethyl)furan (DAF), respectively, and acetone or pyruvate as co-product.

2. Results and Discussion

2.1. Activity of Soluble ATAs towards HMF and DFF

Four different amine transaminases (ATAs) were studied for their activity towards HMF and DFF as substrates with L-alanine as co-substrate: Three (*S*)-selective ATAs from *Vibrio fluvialis* (ATA-Vfl), *Burkholderia multivorans* (ATA-Bmu), and *Silicibacter pomeroyi* (ATA-Spo), and one (*R*)-selective ATA from *Luminiphilus syltensis* NOR5-1B (ATA-Lsy) (Figure S1). As the ATAs used may have different pH profiles for the amination of HMF and DFF, an appropriate pH for all reactions catalyzed by the ATAs should be evaluated for comparison purposes. Therefore, the conversion of both substrates was analyzed at different pH values (pH 6–11 with 1.0-steps, Figure S2) with alanine as a co-substrate. The decrease in the concentration of HMF and DFF was followed spectrophotometrically. All four transaminases appeared to exhibit the same optimum at pH 8.0–9.0 for both substrates, with a very similar pH profile. However, the specific activities of these transaminases differed significantly. While ATA-Vfl and ATA-Bmu showed the highest activities for both substrates, ATA-Spo and ATA-Lsy were less active (Table 1, Figure S2). In addition, the specific activities were generally higher when HMF was used as a substrate compared to DFF, which could be attributed to the two aldehyde groups present in DFF, which require two amination steps.

Table 1. Specific activity of soluble ATAs with HMF and DFF as substrates. The specific activity of soluble ATAs (ATA-Vfl, ATA-Bmu, ATA-Spo, and ATA-Lsy) was determined at different pHs as described in Figure S2 and listed here for pH 8.0 (at which the standard reaction is performed) and 9.0 (pH-optimum for most of the ATAs used). Experiments were performed in triplicate.

Transaminase	Substrate	Specific Activity at pH 8.0 [mU/mg Enzyme]	Specific Activity at pH 9.0 [mU/mg Enzyme]
ATA-Vfl	HMF	105.3 ± 0.3	126.1 ± 0.5
	DFF	113.1 ± 3.1	96.1 ± 0.7
ATA-Bmu	HMF	117.0 ± 1.0	138.4 ± 4.2
	DFF	63.3 ± 1.7	69.1 ± 0.3
ATA-Spo	HMF	48.4 ± 0.1	57.7 ± 0.5
	DFF	39.1 ± 0.8	47.8 ± 1.5
ATA-Lsy	HMF	30.8 ± 0.7	46.1 ± 3.0
	DFF	27.3 ± 0.4	34.8 ± 2.4

2.2. Immobilization of ATAs

Next, the immobilization of ATA-Spo on amine (HA)- and glutaraldehyde-functionalized amine (HA_{GA}) beads was analyzed to establish optimal immobilization conditions, which has already been carried out previously for the other three ATAs [57]. For HA-immobilization, ATAs with the aldehyde tag (encoding the amino acid sequence

CTPSR) [86] were incubated with the formylglycine-generating enzyme (FGE) to convert the cysteine to the unique Cα-formylglycine [87,88], which interacts with the amine functions exposed on the beads to allow site-selective and targeted immobilization. In contrast, non-tagged ATAs were immobilized on HA_{GA} beads by multiple bindings of exposed amino acid residues (especially lysines) [89,90] to the aldehyde functions exposed on the beads. In general, ATA-Spo was optimally immobilized at 22 °C, at a pH of 5.0 (HA_{GA}-) or 7.5 (HA and HA_{GA}-immobilization), with 100 µg (HA_{GA}-) or 150 µg (HA and HA_{GA}-immobilization) enzyme per mg bead, and with a short duration of 4 h (Figure S3). These conditions were similar to those established for the other ATAs used in this study when immobilized on HA- and HA_{GA} beads [57]. Using the optimized immobilization conditions for each ATA, specific immobilization parameters were evaluated in this study (Table 2). The immobilization parameters (i.e., specific activity of the immobilizates, binding efficiency, and activity recovery) of ATA-Vfl, ATA-Bmu, and ATA-Lsy in this study were comparable to those previously reported [57], and the parameters of newly immobilized ATA-Spo immobilization were likewise similar. In general, with regard to the specific activities of the immobilized enzymes, immobilization on HA_{GA} beads (>50 U/mg_{bead}) appeared to be superior to immobilization on HA beads for all (*S*)-selective ATAs studied here. In contrast, the (*R*)-selective ATA-Lsy exhibited higher specific activity when immobilized on HA beads (52 U/mg_{bead}) compared with HA_{GA} beads (34 U/mg_{bead}).

Table 2. Immobilization parameters. To evaluate various immobilization parameters, each transaminase was immobilized on a larger scale (280 mg beads) using the optimized conditions (Table 1), and protein concentration and activity were determined in all solutions. The specific activities of soluble and immobilized ATAs were determined as described in the methods using *rac*-1-PEA as substrate (50 mM Tris pH 8.0, 2.5 mM *rac*-1-PEA, 2.5 mM pyruvate, 0.1 mM PLP, 0.5% DMSO) at 37 °C.

Transaminase	Specific Activity of Soluble Enzyme [a] [U/mg Enzyme]	Bead Type	Specific Activity of Immobilized Enzyme [b] [U/g Bead]	Binding Efficiency [c] [%]	Activity Recovery [d] [%]
ATA-Vfl	4.2	HA	48.6	74.5	10.6
		HA_{GA}	56.3	61.9	9.7
ATA-Bmu	2.0	HA	26.6	97.6	18.2
		HA_{GA}	51.8	71.1	17.7
ATA-Spo	0.8	HA	35.8	92.5	14.2
		HA_{GA}	57.6	93.8	12.4
ATA-Lsy	1.0	HA	52.3	75.3	14.4
		HA_{GA}	33.6	96.2	16.1

[a] The specific activities of soluble ATA-Vfl, ATA-Bmu, and ATA-Lsy were previously determined under equal conditions [57]. The specific activities of the soluble non-tagged ATAs (used for HA_{GA} bead immobilization) did not change by adding the aldehyde tag (used for HA bead immobilization), and their reaction behavior was similar; hence, only one activity is listed. [b] The specific activity of the immobilized enzyme is the observed activity of the immobilized ATA per g of bead support. [c] The binding efficiency is the percentage ratio between the total amount of immobilized enzyme (the protein amount in the starting solution minus the protein amount in the supernatant) and the total amount of protein initially applied for immobilization. [d] The activity recovery is the percentage ratio between the observed total activity of the immobilized biocatalyst and the total activity initially applied for immobilization.

2.3. Amination of HMF and DFF Using Immobilized ATA-Spo in Batch

To analyze the amination of HMF and DFF by an immobilized transaminase, ATA-Spo immobilized on HA_{GA} beads was selected to be used in batch synthesis with L-alanine or isopropylamine as a co-substrate (Figure 1). In general, HMF conversion was much faster than DFF conversion for both co-substrates, which is consistent with the lower activity of soluble ATAs towards DFF. However, it must be considered that DFF undergoes two amination steps via an aminoaldehyde intermediate. Interestingly, the initial reaction rate for HMF and DFF was lower with isopropylamine compared to alanine, which may be attributed to better acceptance of alanine by transaminases, as is often observed [91,92].

However, with increasing reaction time, the conversion of both substrates became more efficient with isopropylamine (i.e., the conversion was faster), and furthermore, both substrates were aminated to a higher extent (88% (HMFA) and 88% (DAF) vs. >99.5% (HMFA) and 99% (DAF) conversion of HMF to HMFA and DFF to DAF with alanine and isopropylamine as co-substrate, respectively). Other reports of transaminases applied in soluble form for these reactions show comparable or lower conversion. Dunbabin et al., reached up to approx. 89% (HMFA) and 70% (DAF) conversion after 24 h using 1-phenylethylamine (5-fold excess) and isopropylamine (10-fold excess) as co-substrate [17]. Interestingly, in this study, 1-phenylethylamine was a better co-substrate for DFF conversion, whereas isopropylamine was better for the conversion of HMF in most other cases. Wang et al. achieved 93.2% HMFA with L-alanine (15-fold excess) [41] and Gao et al. reached a comparable conversion of 97.7% HMFA with D-alanine (24-fold excess) [40], both using whole cells.

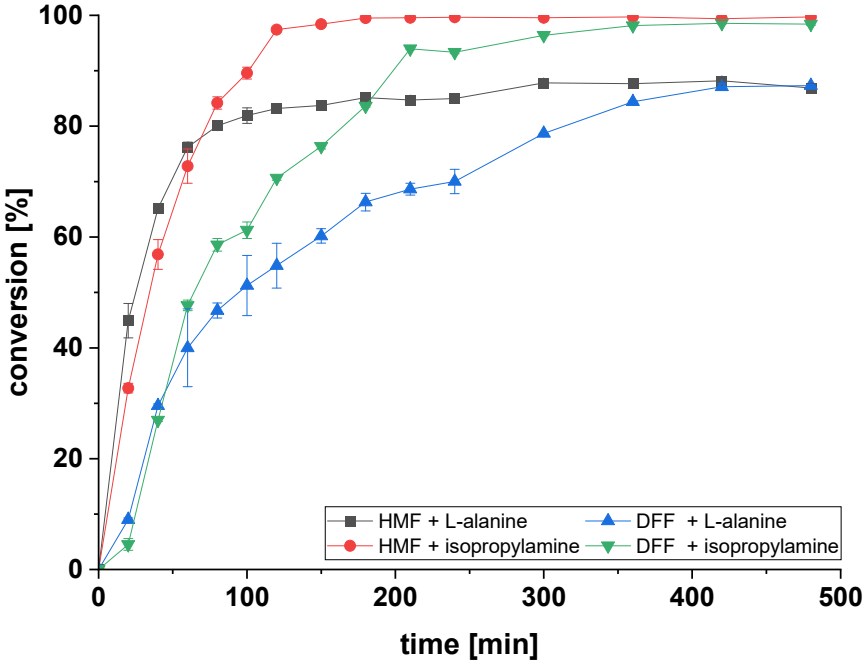


Figure 1. Amination of HMF and DFF by immobilized ATA-Spo in batch reaction. ATA-Spo was immobilized on HA_{GA} beads (5 mg each) and used for the amination of HMF and DFF in 1 mL reaction solution (50 mM HEPES pH 8.0, 13 mM HMF or DFF, 500 mM isopropylamine or L-alanine, 0.1 mM PLP) in 2 mL reaction vessels at 37 °C with shaking. The reaction was followed by the detection of HMF and DFF in diluted supernatant at 283 nm, and the initial concentration was set at 100%. All reactions were performed as duplicates, and the error bars represent the maximum and minimum. (■: HMF and L-alanine; ●: HMF and isopropylamine; ▲: DFF; and L-alanine ▼: DFF and isopropylamine).

The lower conversion in reactions with alanine as co-substrate could be due to product inhibition by pyruvate, which has been observed with various transaminases [93,94], and to an unfavorable equilibrium on the substrate side, which is often observed, for example, in transaminase-catalyzed asymmetric synthesis reactions [95,96]. Product inhibition and unfavorable equilibrium may be counteracted by using isopropylamine as a co-substrate and removing the co-product, acetone, through evaporation, which has already been successfully addressed in other studies [97,98].

2.4. Reusability of Immobilized ATAs for Amination of HMF Using Alanine or Isopropylamine

Immobilization of enzymes offers the major advantage of reusability of the catalysts for multiple reaction cycles. Moreover, in a continuous flow reactor, enzyme immobilization is necessary, and high reusability stability is desirable [99,100]. To analyze the reusability

potential of the immobilized ATAs, repeated cycles of HMF amination with an excess of isopropylamine or alanine as co-substrate were performed for 4 h each at 37 °C.

When alanine was used as the amine donor (Figure 2a), high conversion (>99%) as well as high reusability stability (little to no reduction in conversion) were observed in all cases, except for ATA-Bmu immobilized on HA beads and ATA-Lsy immobilized on HA_{GA} beads. Thus, alanine as a co-substrate seems to be a good choice as an amine donor, as it leads to high conversion and high reuse stability for most of the ATAs studied here. Furthermore, alanine is accepted by most transaminases and is generally considered an environmentally friendly and sustainable chemical. The high reuse stability of ATA-Vfl, ATA-Bmu, and ATA-Lsy was also observed when 1-phenylethylamine (1-PEA) was used as a substrate and alanine as a co-substrate [57]. In contrast, isopropylamine (Figure 2b) had a significant negative effect on the reusability of most immobilized ATAs. The conversion of HMF to HMFA catalyzed by ATA-Vfl gradually decreased with each cycle, dropping from 98% and 90% in the first cycle to 36% and 35% in the fifth cycle for HA_{GA}- and HA-immobilizates, respectively. In the case of ATA-Bmu immobilized on HA_{GA} beads, the conversion decreased from 83% in the first cycle to 63% in the fifth cycle, indicating the destabilizing effects of isopropylamine, but was lower compared to ATA-Vfl. The latter may be due to the generally high operational and solvent stability described for the tetrameric ATA-Bmu [101]. However, when immobilized on HA beads, the conversion was very low (13%) from the first cycle, leading to the assumption that ATA-Bmu is destabilized either by the C-terminally added aldehyde tag or by the C-terminally oriented site-selective immobilization. ATA-Lsy does not appear to accept isopropylamine as an amine donor substrate, as no conversion was observed with either immobilizate. They showed high activity when alanine was used. Interestingly, ATA-Spo immobilized on both HA_{GA} and HA beads yielded the highest conversion of HMF to HMFA using isopropylamine as a co-substrate, with no reduction in conversion observed over all five cycles. Thus, ATA-Spo appeared to accept isopropylamine as a co-substrate best and to have the highest reuse stability compared with the other ATAs studied. In general, various transaminases do not accept isopropylamine at all or accept it poorly, resulting in low conversion of the ketone substrate (here HMF) [102–104]. Consistent with the results shown here, ATA-Spo and ATA-Bmu have shown tolerance to isopropylamine in other studies at higher concentrations as co-substrate for the amination of various substrates, resulting in high conversion of the substrates [92,105] In contrast, ATA-Vfl has been shown not to accept isopropylamine well [106], which explains the rapid decrease in the conversion of HMF with isopropylamine as a co-substrate with each cycle.

Since ATA-Spo immobilized on HA_{GA} beads exhibited the highest stability with respect to its reuse and the use of isopropylamine as a co-substrate, it was selected for further analysis in continuous-flow catalysis despite its lower activity compared to the other ATAs studied.

2.5. Amination of HMF in Continuous Flow Using Immobilized ATA-Spo with Alanine or Isopropylamine

Finally, the performance of ATA-Spo immobilized on HA_{GA} beads in a continuous-flow reactor was investigated. For this purpose, the reaction solution containing 10 mM HMF was passed through the reactor containing the immobilizates (50 mg) at 21 °C with a flow rate of 50 µL/min to achieve a residence time of approx. 4 min in a reactor volume of 236 µL (Figure 3). The column and feed solution were kept dark to avoid the inactivating effect of light on ATA-Spo [49,50]. The protein concentration in the flow-through was analyzed with the Bradford Assay, but no detectable enzyme leaching was observed. The residence time of 4 min was sufficient to achieve a high initial conversion of HMF to HMFA in the first 4 and 24 h when either L-alanine (98% and 95%, respectively) or isopropylamine (75% and 67%, respectively) were used as amine donors, with significantly higher conversion after up to 4 days when L-alanine was used. The latter observation can be attributed to the higher reaction velocity observed before when using L-alanine in batch

synthesis as a co-substrate (Figure 1), leading to a faster conversion in the continuous-flow mode and thus a higher total production of HMFA. To investigate the stability of the immobilized ATA, the flow reactor was run for 12 days, and samples were analyzed every day, showing that the conversion of HMF continuously decreased over time in both cases, reaching 48% and 41% after 12 days when using L-alanine and isopropylamine as co-substrate, respectively. The gradual decrease over time is likely caused by the inactivation of the immobilized enzyme, whose operational stability under the given conditions was not high enough. High amine concentrations, as present in the feed solution, tend to destabilize transaminases [44,107]. The initial conversion was comparable to other studies using alanine and isopropylamine as co-substrate in reductive aminations catalyzed by immobilized transaminase [80,85,108]. Importantly, in other studies, the short- and long-term conversion was optimized by a higher temperature, a longer residence time, and/or a higher amount of immobilizate per reactor volume, which may also increase the conversion of HMF in this study. Using the initial conversion (day 1) of the continuous-flow synthesis of HMFA, a space-time yield of 8 mg $\times$ mL^{-1} $\times$ h^1 was achieved when using either L-alanine or isopropylamine as co-substrate and a half-life of 11 days (using L-alanine) and 9.5 days (using isopropylamine).

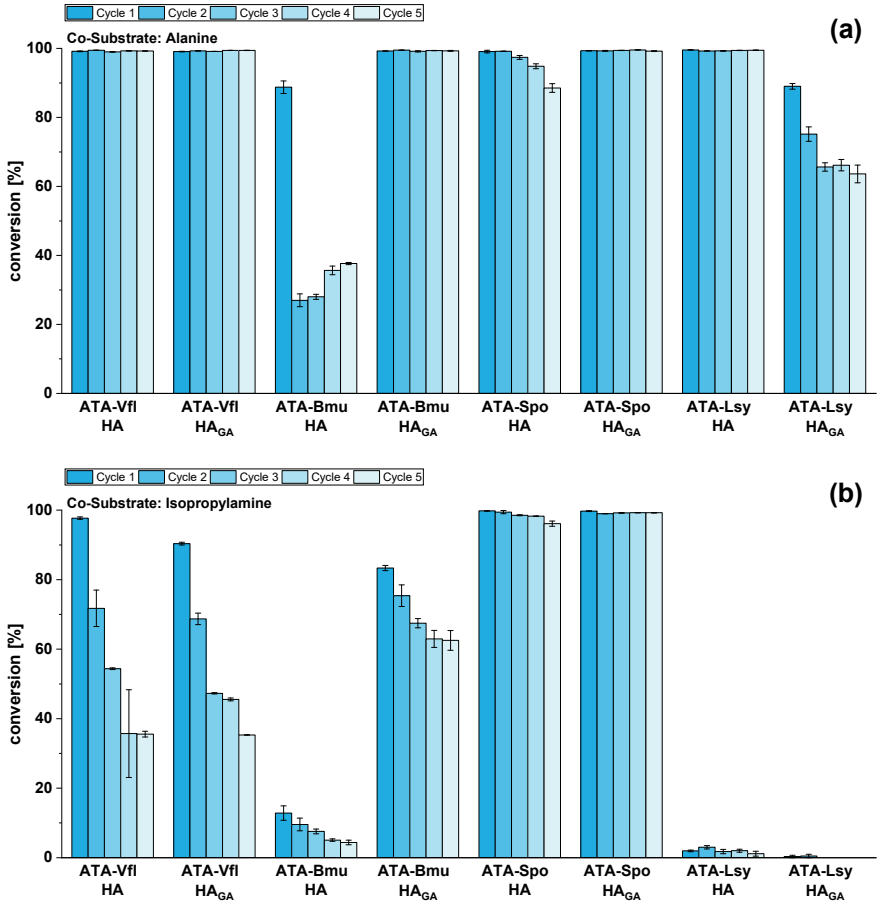


Figure 2. Reusability of ATA-immobilizates for HMF amination. The transaminases (ATA-Vfl, ATA-Bmu, ATA-Spo, and ATA-Lsy) were immobilized separately on HA- or HA_{GA} beads (5 mg each) and used for the amination of HMF to HMFA in five repetitive cycles with alanine (**a**) or isopropylamine (**b**) as the co-substrate. After each cycle, the supernatant was removed, and the conversion of HMF was determined using analytical HPLC as described in the methods. Subsequently, the beads were washed three times with water and incubated with a new reaction solution (1 mL of 50 mM HEPES pH 8.0, 10 mM HMF, 500 mM isopropylamine or alanine (L- or D-alanine for (*S*)- or (*R*)-selective ATAs, respectively), 0.1 mM PLP) for 4 h at 37 °C with shaking. All reactions were performed as duplicates, and the error bars represent the maximum and minimum.

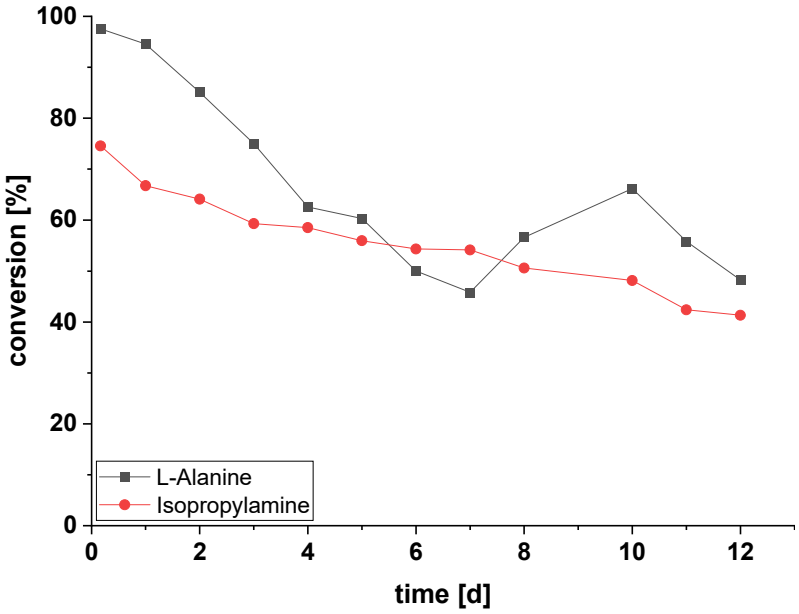


Figure 3. Amination of HMF by immobilized ATA-Spo in a continuous-flow reactor. ATA-Spo, immobilized on 50 mg HA_{GA} beads, was used for the amination of 10 mM HMF to HMFA in a continuous flow with isopropylamine (●) or L-alanine (■) as co-substrate. The reaction solution (50 mM HEPES pH 8.0, 10 mM HMF, 500 mM co-substrate, 0.1 mM PLP) was applied at a constant flow rate of 50 µL/min. The residence time of the reaction solution in the reactor column was approx. 4 min. The reaction was performed at 21 °C, and the conversion was determined by quantification of HMF and HMFA in the flowthrough using analytical HPLC as described in the Materials and Methods.

3. Materials and Methods

3.1. General Information

All chemicals and solvents used were obtained mainly from commercial distributors of analytical grade: Merck (Darmstadt, Germany), VWR (Radnor, PA, USA), Carl Roth (Karlsruhe, Germany), and Thermo Fisher Scientific (Waltham, MA, USA). The amine (ReliZyme^{TM}, HA 403, abbreviated as HA-) beads were purchased in M-size from Resindion S.r.l. (Binasco, Italy) 2,5-diformylfuran (DFF) was chemically synthesized by oxidation of fructose following the literature protocol [37]. The product was obtained with 99% purity, as determined by ^{1}H-NMR in $CDCl_3$.

3.2. Preparation of Glutaraldehyde-Functionalized Amine Beads

The amine beads were functionalized with glutaraldehyde (abbreviated as HA_{GA} beads) exactly as described previously [57].

3.3. Cloning of the Aldehyde-tagged ATA-Spo

Aldehyde-tagged ATAs (after FGly generation; see below) were used exclusively for immobilization on the HA beads. The addition of the aldehyde tag to ATA-Vfl, ATA-Bmu, and ATA-Lsy has been described previously [57,86], whereas the generation of the aldehyde-tagged ATA-Spo was performed in this study according to the protocols used for the other ATAs. Briefly, the coding sequence of ATA-Spo was amplified with add-on primers (forward: aaggagatatacatatg *agcctggcgaccattacg*, reverse: gagtacataaactagcact ta *cttcagatttcatcagaccctg*; underlined sequences: complementary sequences for the subsequent overlap extinction (OE) PCR); italic sequences: sequences for the amplification of the coding sequence of ATA-Spo) by add-on PCR (analogous to that described previously with an annealing temperature in the first 4 cycles of 63 °C). Subsequently, the amplicons were used as megaprimers in OE-PCR (exactly as described previously) to insert the sequence into the

previously generated plasmid pET24b-X-CTPSR-H6 (at position X with the C-terminally aldehyde tag (CTPSR) and the His_6 tag (H6) [86].

3.4. Expression and Purification of Enzymes

The expression of Δ72-hFGE was performed as described by Peng et al. [87]. The purification of Δ72-hFGE and the expression of the tagged and non-tagged ATA-Vfl, ATA-Bmu, and ATA-Lsy were exactly performed as described previously [57]. Expression and purification of the tagged and non-tagged ATA-Spo were performed analogously as for the other ATAs.

3.5. Conversion of the Aldehyde Tag

The conversion of cysteine to Cα-formylglycine within the aldehyde tag was catalyzed by Δ72-hFGE. Therefore, Δ72-hFGE was added to the reaction solution (50 mM Tris pH 8.0, 0.1 mM PLP, 3 mM DTT) containing the tagged ATA in a molar ratio of 1:30 (Δ72-hFGE:ATA) and incubated for 4 h at 37 °C. Conversion was verified by fluorescent labeling with Alexa Fluor™ 488 Hydroxylamine, carried out exactly as previously described without further purification [57].

3.6. Immobilization of ATAs on the Solid Supports

The establishment of the immobilization conditions and the final immobilization of the ATAs on HA- and HA_{GA} beads were exactly performed as described previously [57].

3.7. Activity Assay of Soluble and Immobilized ATAs

The specific activity of the ATAs was determined differently for each substrate. In the case of 1-PEA, the product, acetophenone, was detected at 245 nm and quantified using the extinction coefficient of 12 mM^{-1} cm^{-1} [109]. When HMF or DFF were used as substrates, the decrease in absorbance of the substrates was detected at 283 nm and quantified using the extinction coefficients of 14.922 mM^{-1} cm^{-1} (HMF) and 10.610 mM^{-1} cm^{-1} (DFF) (Figure S4). Using soluble enzymes, the reactions were performed in UV-microtiter plates (UV-Star®, Greiner Bio-One, Kremsmünster, Austria) containing the stated amounts of ATA and a reaction volume of 150 µL (50 mM Tris pH 8.0, 0.5% DMSO, 0.1 mM PLP) with the respective substrates (*rac*-1-PEA with pyruvate, HMF or DFF with isopropylamine or alanine (L- or D-alanine for (*S*)- or (*R*)-selective ATAs, respectively) at the respective indicated concentrations, and followed at the respective wavelength in the TECAN reader (Spark™ 10M, Männedorf, Switzerland) at 37 °C. In the case of the pH-screen, different buffers were used instead of the one standard buffer mentioned above: 50 mM phosphate buffer pH 6.0, 50 mM Tris buffer pH 7.0–9.0, or 50 mM CAPS buffer pH 10.0–11.0. Specific activity was always determined at the initial reaction rate by using the average of 1 min intervals in the first few minutes (generally 5–10 min). When using the immobilized enzymes, 5 mg (the standard amount of beads unless stated otherwise) of the immobilizates were shaken with 1.5 mL (the establishment of the immobilization) or 1.0 mL of reaction solution (as indicated in each case) at 37 °C for 2 min. Subsequently, the analytes in the diluted supernatant were determined at the respective wavelengths in the TECAN reader. In the case of batch synthesis (Figure 1), the reaction was followed for 480 min, while the conversion was followed by detecting the products as mentioned. The reactions catalyzed by immobilized ATAs were performed in sealed 2 mL reaction tubes to avoid evaporation of the products (i.e., acetophenone) [110]. One unit was defined as the amount of soluble or immobilized enzyme that produced 1 µM of the product in one minute at the mentioned temperatures. All reactions were performed in duplicate and always started by adding the reaction solution.

3.8. Batch Reusability Study

To investigate the reuse potential of each immobilized ATA in reductive amination of HMF, 5 mg of each immobilizate were incubated in 1 mL reaction buffer (50 mM HEPES

buffer pH 8.0, 10 mM HMF, 0.1 mM PLP, and 500 mM amine donor (isopropylamine or L- or D-alanine for (*S*)- or (*R*)-selective ATAs, respectively) at 37 °C for 4 h in 1 mL reaction tubes under vigorous shaking. After each cycle, the reaction solution was removed, the beads were washed with water, and they were used for another cycle. All experiments were performed in duplicate and repeated for a total of five cycles. The concentration of HMF and HMFA was analyzed using HPLC.

3.9. Continuous Flow Catalysis

ATA-Spo immobilized on 50 mg HA_{GA} beads was added to a glass tube (inner diameter 10 mm, total length 100 mm, approx. height of bead layer 3 mm, approx. bead volume 0.235 mL), which was then filled with feed solution and attached to the pump (Metrohm 665 Dosimat, Herisau, Switzerland). The feed solution (50 mM HEPES buffer pH 8.0, 10 mM HMF, 0.1 mM PLP, and 500 mM amine donor (isopropylamine or L-alanine)) was pumped through the tube with a flow rate of 50 µL min^{-1}. The tube and the feed solution were wrapped in aluminum foil to exclude negative influences from light [49,50]. Samples of the flow were taken every 24 h and analyzed using HPLC.

3.10. HPLC Analysis

To quantify the concentration of the substrates and products, analytical HPLC analyses were performed using a Waters 2695 Separations Module (Milford, MA, USA) with a Waters 2996 Photodiode Array Detector and a Supelco Discovery® (St. Louis, MO, USA) C18 column (15 cm × 4.6 mm). Therefore, 1 mL samples of batch and continuous flow catalysis experiments were analyzed using an isocratic method (98% acetonitrile + 2% ddH_2O + 0.1% trifluoroacetic acid) at a constant flow of 1 mL min^{-1}. Peaks were detected using a DAD detector at 210 nm. Chromatograms in Figure S5.

3.11. Determination of Protein Concentration

For the determination of the total protein concentration in protein solutions, the Protein Assay Dye Reagent Concentrate from BioRad (Hercules, CA, USA) (Art.5000006) was used with bovine serum albumin as the calibration standard.

4. Conclusions

In this study, the biocatalytic reductive amination of HMF and DFF using amine transaminases (ATAs) was performed. Four ATAs that exhibited activity towards HMF and DFF were identified. Different pH values were tested for the ATAs, revealing that slightly basic conditions led to the highest specific activity. Following, all four ATAs were successfully immobilized on solid supports, with the (*S*)-selective ATAs (ATA-Vfl, ATA-Bmu, and ATA-Spo) showing the highest specific activity when immobilized on glutaraldehyde-functionalized amine (HA_{GA}) beads via multi-attachment binding (56, 52, and 58 U/g_{bead}, respectively). On the other hand, immobilization on amine beads via site-selective, oriented binding yielded the highest specific activity of the (*R*)-selective ATA (ATA-Lsy, 52 U/g_{bead}). Almost all of the immobilized ATAs demonstrated efficient reusability when alanine was used as the amine donor for the reductive amination of HMF. However, when isopropylamine was used, only ATA-Spo immobilized on both supports and ATA-Bmu immobilized on HA_{GA} beads revealed high reuse potential. High conversion rates were achieved using ATA-Spo immobilized on HA_{GA} beads in the batch synthesis of HMFA and DAF with alanine as an amine donor (87% and 87%, respectively) and almost complete conversion with isopropylamine as an amine donor (>99 % and >98 %, respectively). Finally, the reductive amination of HMF in continuous-flow mode using ATA-Spo immobilized on HA_{GA} beads was conducted. High conversion was achieved even after 12 days (48% and 41% when alanine and isopropylamine were used as amine donors), with a half-life of 11 days with L-alanine and 9.5 days with isopropylamine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal13050875/s1, Figure S1: Amination of HMF by soluble ATAs; Figure S2: Relative activity of different ATAs at different pH with HMF and DFF as substrate; Figure S3: Analysis of various immobilization conditions for ATA-Spo; Figure S4: Characterization of HMF, HMFA, DFF, and DAF., Figure S5: HPLC Analysis of HMFA and HMF.

Author Contributions: Conceptualization, T.H., L.M.M., L.M.v.L. and P.B.; methodology, T.H., L.M.M. and J.L.; validation, T.H. and L.M.M.; formal analysis, T.H. and L.M.M.; investigation, T.H., L.M.M. and J.L.; data curation, T.H. and L.M.M.; writing—original draft preparation, T.H. and L.M.M.; writing—review and editing, all authors; visualization, T.H. and L.M.M.; supervision, G.F.v.M., L.M.v.L., M.H., U.T.B. and P.B. All authors have read and agreed to the published version of the manuscript.

Funding: This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 860414.

Data Availability Statement: Data is contained within the article or Supplementary Materials.

Acknowledgments: The authors would like to acknowledge Chiara Danielli (ViaZym B.V.) for synthesizing DFF and her expertise on enzyme immobilization; Nils Berelsmann (University of Bielefeld) for his help with the HPLC and UV/VIS measurements; and Kerstin Glüsenkamp (University of Bielefeld) for her technical support.

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

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