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A Rapid Selection Procedure for Simple Commercial Implementation of ω-Transaminase Reactions

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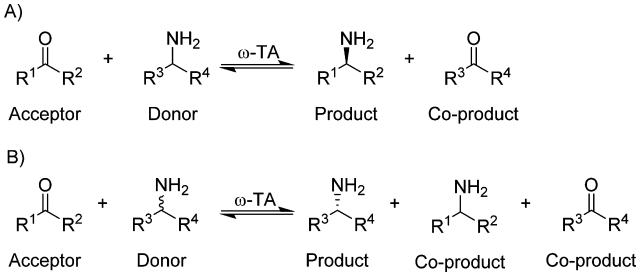
ABSTRACT: A stepwise selection procedure is presented to quickly evaluate whether a given ω-transaminase reaction is suitable for a so-called "simple" scale-up for fast industrial implementation. Here "simple" is defined as a system without the need for extensive process development or specialized equipment. The procedure may be used when investment in intensive process development cannot be justified or when rapid execution is paramount, for applications such as small singular batches. The three-step evaluation procedure consists of: (1) thermodynamic assessment, (2) biocatalyst activity screening, and (3) determination of product inhibition. The method is exemplified with experimental work focused on two products: 1-(4-bromophenyl)ethylamine and (*S*)-(+)-3-amino-1-Boc-piperidine, synthesized from their corresponding pro-chiral ketones each with two alternative amine donors, propan-2-amine, and 1-phenylethylamine. Each step of the method has a threshold value, which must be surpassed to allow "simple" implementation, helping select suitable combinations of substrates, enzymes, and donors. One reaction pair, 1-Boc-3-piperidone with propan-2-amine, met the criteria of the three-step selection procedure and was subsequently run at 25 mL scale synthesizing (*S*)-(+)-3-amino-1-Boc-piperidine at concentrations up to 75 g/L. However, the highest product yield (70%) was obtained at a lower substrate concentration of 50 g/L.

1. INTRODUCTION

Over the past decade, biocatalysis has become an established and useful complement to conventional chemical catalysis for the synthesis of fine chemicals. Most often, biocatalytic methods have been selected due to exceptional selectivity (regio- and/or enantioselectivity).[1] In fact, the majority of industrially applied biocatalytic reactions today yield optically pure chiral products that are used in the fine chemical industry as building blocks for agrochemicals and pharmaceuticals.[2] In particular, biocatalytic transamination chemistry has been identified as one of the key emerging areas for the pharmaceutical industry[1,3] as a means of producing optically pure chiral amines. This paper focuses on the biocatalytic synthesis (and resolution) of chiral amines of high optical purity using ω-transaminase (ω-TA) (E.C. 2.6.1.18), which is a type of amino transferase. ω-TA was chosen as a catalyst for this work due to its outstanding stereoselectivity and broad ketone substrate repertoire. Two ω-TA-catalyzed paths are available toward optically pure chiral amines, using either asymmetric synthesis or kinetic resolution. Although the latter is challenged by a maximum 50% yield,[4−6] both are considered as potential options for the "simple" scale-up.

ω-TA catalyzes an amino transfer reaction, illustrated in Scheme 1. Briefly, in the synthetic direction (Scheme 1A) the amino donor (an amine), and the amino acceptor (a prochiral ketone), here referred to simply as the "donor" and "acceptor", respectively, react with the enzyme in a sequential fashion producing the desired target chiral amine product and a coproduct. Detailed descriptions of the sequential ping-pong bi-bi enzymatic reaction mechanism can be found elsewhere.[7,8] In the resolution reaction (Scheme 1B) the same reaction takes place, but now the amino donor is added as a racemic mixture.

Scheme 1. Examples of Potential ω-TA Reactions Using (A) a Synthetic Route and (B) a Resolution Route



Through reaction therefore, one isomer is left unreacted, which becomes the desired optically pure product.

The amino moiety alone is transferred between the two starting substrates, and therefore in the synthetic direction, the molecular structure of the chiral product will be determined by the structure of the acceptor molecule. This means that the donor molecule can be freely chosen, since it neither affects the target product structure nor the stereoselectivity. In principle therefore, a plethora of possible donors could be chosen, although in the scientific and patent literature only a handful of amine donors have been reported. The authors have recently proposed a novel quantum mechanical method to determine the free energy of compounds and hence the thermodynamic feasibility of using novel amino donors for this reaction type, irrespective of kinetic considerations.[9] This along with a wider implementation of this technology in the future is likely to lead to a broader range of different amino donors.

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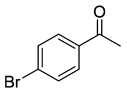
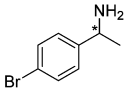
Table 1. Compounds Used in This Study[a]

Reactants	Acceptor ketones		Amino donors	
				
	1	2	3	4
Products	**Target chiral amine products**		**Co-products**	
				
	5	6	7	8

[a]Acceptor ketones: 4-bromoacetophenone (1), 1-Boc-3-piperidone (2); amino donors: propan-2-amine (3), 1-phenylethylamine (chiral) (4); target chiral amine products: 1-(4-bromophenyl)ethylamine (chiral) (5), 3-amino-1-boc-piperidine (chiral) (6); coproducts: acetone (7) and acetophenone (8).

Despite the interest in such reactions, they are often demanding to implement on an industrial scale due to frequent thermodynamic and kinetic challenges.[10] While many technical solutions are available to overcome these challenges, the proposed solutions are frequently complex and often require significant process development time. Indeed, for some applications, a fast and simple process development is not only desirable but may be essential for commercial success. In these cases, it will be more important to rapidly develop a simple process, than to obtain an economically optimal process. Such situations include pharmaceutical synthesis in the early phases of clinical testing and other cases such as small singular batches, where investment in extensive process development cannot be justified. Using this logic, and from a knowledge of the properties of a given ω-transaminase-catalyzed reaction and the available enzymes to catalyze the reaction, we reasoned that it should be possible to categorize a particular reaction as "complex" (requiring extensive development) or "simple" (with easy implementation and scale-up). We therefore suggest that an evaluation method allowing the identification and selection of "simple" reactions (and eliminating the "complex" ones) would prove a valuable tool for process chemists.

The scope of this manuscript is therefore to present a stepwise decision-making procedure to quickly identify if a "simple" scale-up is feasible for a given reaction. Hence, solutions such as biocatalyst modification by protein engineering,[10] amino donor recycling,[11,12] and equilibrium shifting methods[13,14] have not been considered here. The three-step decision-making procedure involves an evaluation of: (1) thermodynamics, (2) biocatalyst activity, and (3) product inhibition. Each step is evaluated against a threshold value, which must be met in order to identify a given case as suitable for "simple" implementation.

2. RESULTS

In order to exemplify the method, experimental data on two chiral target products were evaluated, 1-(4-bromophenyl)-ethylamine (**5**) and (*S*)-(+)-3-amino-1-Boc-piperidine (**6**). These compounds were selected because both products are commercially attractive and additionally biocatalytic transaminations to synthesize both **5**[15] and **6**[11,16] have been reported previously. In these reactions optical purity was necessary and evaluated, but a specific stereoisomer was not required. The prochiral ketone substrates, the amino acceptors, 4-bromoacetophenone (**1**) and 1-Boc-3-piperidone (**2**), corresponding to the products above, were reacted with two possible donor molecules propan-2-amine (**3**) and 1-phenylethylamine (**4**) (Table 1). Both amino donors have frequently been used in a wide variety of biocatalytic transamination. Between them, they represent different classes of donor. For instance, donor **3** serves as an inexpensive achiral donor. In contrast amino donor **4** is a more costly chiral compound which has also been reported to be inhibitory[17] with downstream processing complications due to separation issues when the product shares structural similarity. However, donor **4** also offers a significant thermodynamic advantage, since the carbonyl coproduct, acetophenone (**8**) formation is highly favorable (Table 1). Academically, a more common amino donor that has often been reported is the use of alanine (or pyruvate for the resolution reaction).[4,18] We have previously shown that the thermodynamics using this donor very strongly favors the reverse resolution reaction,[19] and therefore we have not considered this further in this work.

2.1. Method Development. In order to enable rapid evaluation, the three selection criteria are each assigned a threshold value, which must be met to enable implementation of a simple scale-up. The proposed procedure is outlined in Figure 1. In the figure, full lines indicate that the reaction has met (green lines) or failed (red lines) the individual criteria. Likewise, dashed lines indicate an alternative strategy by

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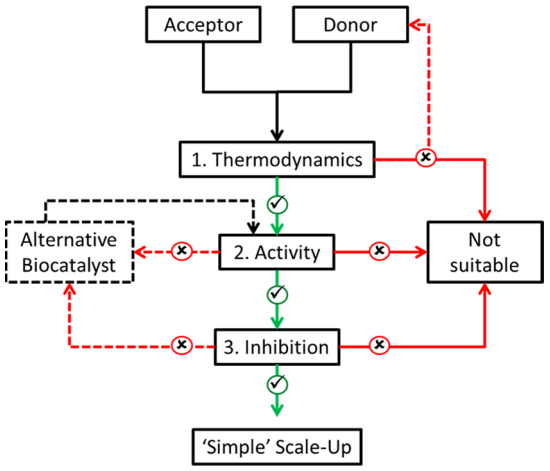

Figure 1. Decision making procedure for a simple scale-up. Green lines marked with a check mark or red lines marked with an X indicate if a given criterion is met or not met, respectively. Dashed lines and boxes indicate options for reassessment if a criterion is not met. Each criteria has cut off values for simple implementation. 1. The thermodynamic criteria is meet when K_{eq} is less than 0.02 (resolution reactions) or greater than 1 (synthetic direction). 2. The activity criterion requires a specific activity greater than 0.05 g/g/h. 3. The inhibition criteria is met at less than 50% activity loss, with 5% of target concentration product present. Possible remediation options, if a given criterion for a simple scale-up is not met, can be to consider an alternate amino donor or test an alternative biocatalyst (dashed lines).

adjusting one of the variable reaction components (the amino donor or the biocatalyst). The threshold values for each criterion are also indicated in the legend of Figure 1, the justification for which is given in the following section.

2.1.1. Thermodynamic Assessment. Unfavorable thermodynamics presents one of the main barriers to the implementation of the transaminase−catalyzed reactions on an industrial scale.[4] The thermodynamic equilibrium constant (K_{eq}) of the reaction is important since it determines the maximum reaction yield for a given concentration of substrates. Thus, we reasoned it is one of the most important parameters for determining the optimal process configuration.[6,13,20] For this reason, we suggest the first step in the procedure should be to determine if a candidate reaction has a suitable thermodynamic equilibrium constant to make a "simple" scale-up feasible.

In the synthetic mode, thermodynamic feasibility is here defined as a K_{eq} above 1.0, since lower values of K_{eq} would require a high excess (more than 20-fold) of the amino donor to obtain sufficient reaction yields (95% or higher), for eventual industrial implementation. Use of such an excess makes the reaction costly and practically difficult to carry out at high substrate concentrations. In a similar way, we reasoned that for reactions with a low K_{eq}, a kinetic resolution would be a better choice for the reaction. On the other hand, the resolution reaction requires more stringent conversion requirements since the separation of the amine product from the unreacted half of the racemic donor starting material is of course quite challenging. Hence we have chosen a K_{eq} threshold of 0.02 in the resolution direction, meaning only values lower than this are suitable for a simple scale-up.

In this work, the concentration-based equilibrium constant was experimentally determined using a previously described method.[20] Since the value is obtained for comparative purposes, practical (rather than standard) conditions were used, meaning it is more accurate to describe the constant as "apparent", K_{eq}^{app}. In principle to save time as an alternative to experimental measurement, in silico methods could be used to estimate such values, although the accuracy is perhaps questionable. Here the K_{eq}^{app} for the two chiral amine products **5** and **6** were measured experimentally using the two donors **3** and **4**, as described above. The K_{eq}^{app} for the four reactions (Table 2) varied by a factor of 10^4 from the most challenging pair, **1** and **3**, at 0.025 to the most favorable pair, **2** and **4**, which had a K_{eq}^{app} of 450, in the synthetic direction. Thus, thermodynamics is indeed highly variable between the four selected reaction pairs. After applying the threshold criteria one of the two products, **5**, was eliminated from further investigation. This may indicate that highly conjugated aryl compounds are not suitable for simple scale up and should be assisted by other process technologies and strategies. For example, it has been reported that one of the compounds we have used as a donor here **4**, could also synthesized and successfully scaled in combination with in situ product removal, alleviating both the thermodynamic and inhibitory strains.[14] None of the reaction pairs evaluated here was found suitable for the resolution reaction, although alanine, the amine donor often found most suited for resolutions reactions was not tested as discussed previously.[19]

Table 2. Experimental Values for K_{eq}^{ap}

	donors	
acceptors	3	4
1	0.025[a]	0.5
2	32	450

[a]Data previously reported in ref 13.

Clearly it is possible to carry forward more than one amine donor to the subsequent evaluation steps, although this is not helpful for the procedure, which aims to focus effort on those cases with the biggest chance of simple scale-up success. In this case, due to the low cost and high water solubility, amine donor **3** was selected for further evaluation.

2.1.2. Biocatalyst Activity Screening. No matter how favorable the thermodynamics, without sufficient activity the reaction will not be completed in a reasonable time, and issues like enzyme inactivation may arise. Hence, the next step of the procedure is to find a suitable biocatalyst with sufficient activity. Candidates for biocatalyst screening can be obtained from commercial screening kits or in-house enzymes. For the "simple" scale-up, strategies such as protein engineering are not considered. Low activity of an enzyme preparation will negatively impact downstream processing, by adding extra proteinaceous material which impedes product recovery. Therefore, the maximum biocatalyst loading was set to 10% v/v irrespective of the biocatalyst formulation. Additionally, product concentration should be in the range of ≥50 g/L[6,10] to assist downstream product recovery. Finally, due to biocatalyst stability concerns, we reasoned it necessary to complete the reaction within 96 h. On this basis, we calculated a minimal biocatalyst specific activity (sometimes termed "biocatalyst productivity"), as a threshold value for the "simple" of 0.05 g/g/h (g product/g biocatalyst/hour).

For this case study a small screen with four enzymes was conducted, using the reactant pair **2** and **3** selected from the previous section. In this screen four selected enzymes were tested, two of which were known to be (*R*)-selective and two

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(*S*)-selective. We reasoned that for this case study the particular stereoselectivity of the enzyme did not influence the overall procedure. Additionally, since this screen was conducted with an achiral amine donor and the pro-chiral ketone, the selectivity of the enzyme would not affect the reactivity with these substrates. The screen showed a large variation between the least and most reactive candidates (Table 3). Details of the

Table 3. Specific Activities Obtained with Reactant Pair 2 and 3, with Selected Enzymes

enzyme	selectivity	specific activity (g/g/h)
Ars-ωTA	S	0.048
Tar0	R	0.003
Tar1	R	0.012
ATA 47	S	0.054

individual enzymes (ATA-47, Tar0, Tar1, and Ars-ωTA) are given in the experimental section of the paper. Tar0 was found to give a specific activity of 0.003 g/g/h, whereas the best candidate (ATA-47) gave a 20-fold higher value of 0.054 g/g/h. ATA-47 was therefore carried to the next step. Likewise the enzyme Ars-ωTA had a high specific activity of 0.048 g/g/h, close to the threshold value.

2.1.3. Determination of Product Inhibition. The final step of the procedure considers product inhibition of the enzyme, which due to the requirement for high product concentrations (50 g/L) in industrial processes,[2] is a frequent hurdle for process intensification of enzyme reactions in general, and ω-TAs in particular.[14] Hence, we set the threshold value here at a 50% reduction in reaction rate in the presence of 2.5 g/L product, under the assay conditions used here (see Experimental Section). Here only product inhibition is assessed, since substrate inhibition can relatively easily be overcome by substrate feeding.

In order to experimentally test for product inhibition, the initial reaction rate of ATA-47 was measured using 100 mM **3** and 10 mM **2**, in the presence of various concentrations of the product **6**, up to 10 mM. Importantly, the substrate concentrations were chosen to avoid limiting the reaction by thermodynamic constraints. Inhibition was observed with 10 mM product and amounted to a 10% initial rate reduction, compared to initial conversion rates in the absence of product. Initial conversion rates were assumed when less than 10% of limiting starting material was converted.

2.1.4. Discussion. First, with respect to thermodynamics, the procedure enables the elimination of unfavorable cases. Clearly each donor or acceptor molecule has an associated free energy which contributes to the net thermodynamics of a given reaction. In this way for instance a comparison of the equilibrium constants of two reactions (with different acceptors, but using the same donor) can be used to interpret the effect of changing acceptors. In an analogous way, one could determine the K_{eq} for a given acceptor with one donor and extrapolate the K_{eq} to other donors with the same acceptor, given one knows the difference in ΔG between the reactions, as discussed elsewhere.[19]

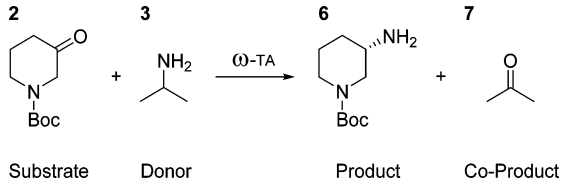
Second, the biocatalyst activity is assessed, since low activity will have drawbacks in the form of low space-time yields and may prevent the reaction from going to completion due to enzyme deactivation. One solution would be to apply high biocatalyst concentration, but these may negatively impact downstream processing by hindering product recovery. Thus, the threshold for the enzyme is defined as minimum specific activity, which for the "simple scale-up" was set at 0.05 g/g/h. Biocatalyst recycle was not considered for the simple scale-up.

Finally a determination of product inhibition is carried out. This is a frequent hurdle for process intensification of ω-TA's,[14] due to the high product concentrations (50 g/L) required to simplify the product recovery.[2] In contrast to the high concentration intensity of commercial processes, enzymes are designed to work under physiological (dilute) conditions. This frequently leads to process intensification challenges with biocatalytic reactions. For example transaminases display a ping-pong bi-bi reaction mechanism, with two sequential half reactions,[8] and this type of reaction mechanism is often plagued by inhibition from competitive dead-end complexes of products bound to the *apo*-enzyme or the incorrect form of the *holo*-enzyme. Hence, understanding the inhibition profile of a potential product is vital in evaluating the possibility of a simple scale-up. As such, we advocate that, if severe inhibitory effects are observed with low product concentrations, it implies a high risk of inhibition under process scale concentrations.

The three-step evaluation method has been successfully applied to a case study, and one reaction pair with one biocatalyst was deemed suitable for "simple" scale-up.

2.2. Intensification and Scale-Up. In the previous sections, the selection procedure for a simple scale-up toward the synthesis of **6** identified acceptor **2** with donor **3** (Scheme 2) using ATA-47 as suitable. In the event ATA-47 was

Scheme 2. Synthetic Transaminase Reaction Carried Out[a]



[a]Compounds: 1-Boc-3-piperidone (**2**), propan-2-amine (**3**), (*S*)-(+)-3-amino-1-Boc-piperidine (*S*)-(+)-**6**), acetone (**7**).

substituted by ArS-ωTA since the difference in activity was negligable and the latter enzyme has been reported to have excellent stereoselectivity.[21,22]

2.2.1. Reaction Optimization: pH and Donor Loading. Prior to scale-up, a small optimization study was undertaken to evaluate if reaction rates could be enhanced by simple optimization within the biocatalyst stability range. A range of pH and donor loadings was explored in an attempt to improve kinetics, with both short (0.5−2 h) and long (18 h) reaction times; the latter time point was chosen to investigate enzyme stability under the given conditions.

The rate dependency on pH was tested between pH 7 and 9, with 40 mM acceptor and 500 mM donor (Figure 2). Other studies have found up to 40% variation in yield in this pH range for similar reactions.[23] Here the fastest reaction rates were identified at pH 9 for all time points. The greatest difference was found in the 18 h reaction times, where average reaction rates are 45% faster at pH 9 compared with pH 7, indicating that this is the best pH, within the pH range tested, with respect to kinetics, and that the enzyme is more stable under these conditions. Since the pK_a of the amine donor **3** is 10.6,[24] meaning a higher pH would render a higher fraction of the substrate uncharged and thus reactive, in principle operating at a higher pH would therefore be beneficial from the perspective

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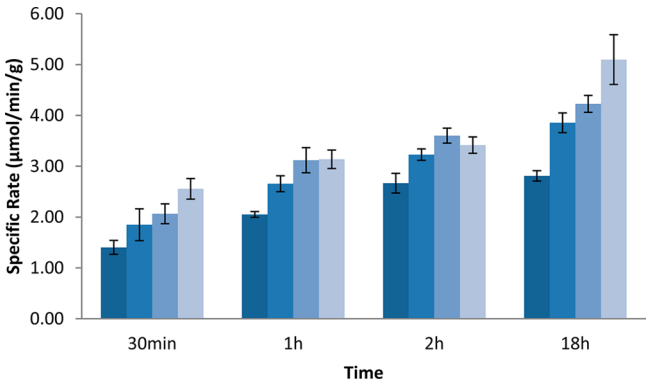

Figure 2. Specific rates measured at four reaction time points at four different reactions, each carried out at different pH values (pH 7.0, pH 7.5, pH 8, and pH 9 from left to right at each time point, respectively).

of the reaction rate. Nevertheless, in this study we limited the pH range to keep the study simple and manageable, consistent with the philosophy of this work, and therefore did not test the reaction at higher pH values than 9.

Furthermore, the same method was used to determine optimal donor loading. Donor concentrations could potentially be limiting, dependent upon K_M[12]. Clearly an excess concentration of the donor (over acceptor) could be used which might also drive the equilibrium.[23,25] This was tested experimentally but at all concentrations tested, the rate was unaffected by donor concentration (Figure 3), suggesting a K_M beneath 100 mM. For subsequent experiments 1 M **3** was used.

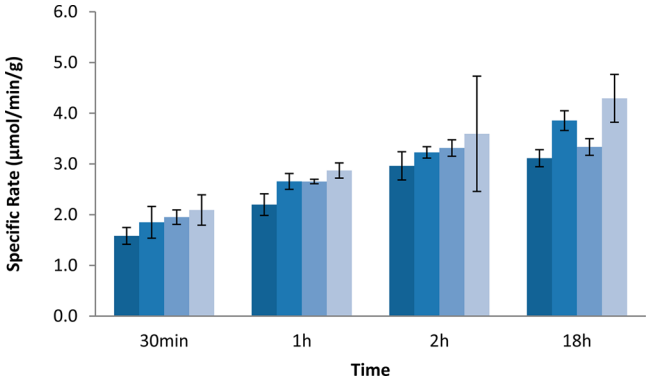

Figure 3. Specific rates found at four time intervals at four donor concentrations (0.1, 0.5, 1, and 2 M from left to right at each time point, respectively) used to investigate the optimal donor loading for the reaction.

2.2.2. Reaction Intensification. As indicated above a viable scale-up depends on reaction intensification (i.e., the synthesis of high product concentrations).[10] This is important in the simple scale-up because too low a concentration will add volume to the reaction and thus complicate the process. The reaction of **2** and **3** using Ars-ωTA was therefore intensified by increasing the substrate concentration up to 75 g/L. Three reactions were done in scintillation vials at concentrations of 25, 50, and 75 g/L. The reactions proceeded smoothly (Figure 4) at both 25 g/L and 50 g/L but not at 75 g/L, the latter most likely due to mass transfer limitations from low solubility and decomposition of the starting material in aqueous conditions. The latter was further investigated and confirmed (data not shown). To the best of our knowledge no other study has

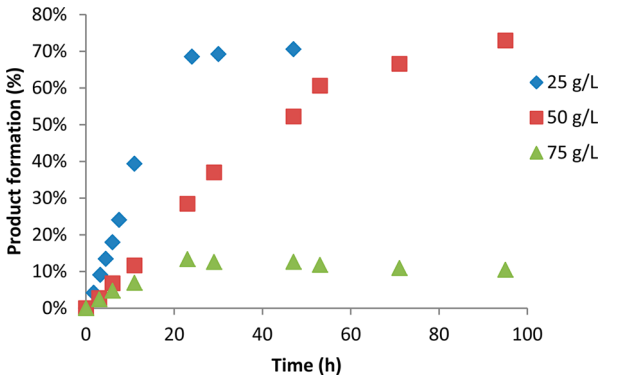

Figure 4. Reaction profile over 96 h with initial substrate concentrations of 25, 50, and 75 g/L.

investigated the stability of this compound in water, either for biocatalysis[11] or chemical catalysis. In the 25 and 50 g/L reactions final conversions of acceptor **2** to chiral amine target **6** of 70% were observed. Figure 5 shows that the initial reaction rates are similar at all substrate concentrations tested, indicating that the reaction is not kinetically controlled (above K_M).

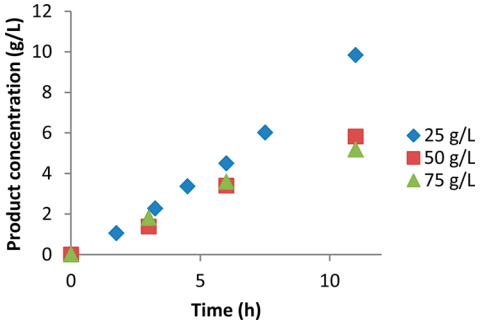

Figure 5. Initial product formation for the first 12 h of the reaction with initial substrate concentrations of 25, 50, and 75 g/L.

2.2.3. Product Identification. Finally, the reaction was run at 25 mL scale for 96 h to isolate product. At 50 g/L substrate concentration the final reaction composition was analyzed to contain 89% **6** and 11% **2** (with an isolated product yield of around 70%). This composition is in excellent agreement with that found in the 50 g/L 1 mL scintillation vial experiment, which gave 91% target chiral amine **6** and 9% ketone **3**, after 96 h.

3. CONCLUSION

A simple stepwise procedure has been described, to facilitate the selection of suitable substrate-donor-enzyme combinations to allow so-called "simple" scale-up. Each step in the procedure has a threshold value which must be met to allow simple implementation. We believe that this method will prove useful both to select good candidates for this technology and to eliminate those that may require further development. A simple case study was used to illustrate the power of the procedure, sequentially eliminating unsuitable substrates, donors, and enzymes. Beyond this case study, we furthermore suggest that analogous procedures could be used for the evaluation of other "simple" biocatalytic processes.

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4. EXPERIMENTAL SECTION

4.1. Materials. Three plasmids encoding the enzymes, Tar0, Tar1, and Ars-ωTA, were kindly provided by Professor NJ Turner (University of Manchester, Manchester, UK). Tar0 encoded the ω-transaminase from *Arthrobacter* sp. KNK168 (Sequence 2 from US 7169592) inserted between the *Nde* I and *Xho* I (with C-terminal His tag) site of pET21a (Accession number ABN35871). Tar1 encoded ω-transaminase from *Arthrobacter* sp. KNK168 (Sequence 110 from US 8293507, Tar1) inserted between the *Nde* 1 and *Xho* 1 (with N-terminal His tag) site of the pET16b (Accession number AFX11601). Ars-ωTA encoded mutated ω-transaminase from *Arthrobacter citreus* (Sequence 16 from US 7172885,) inserted between the *Nde* 1 and *Xho* 1 (with C-terminal His tag) sites of the pET21a (Accession number ABN37907). The commercial enzyme ATA-47 (30902-2; activity 0.41 U/mg; batch LH1-01-02) was purchased from c-LEcta GmbH (Leipzig, Germany).

Deionized water (18 Ω) was used for all experiments. All chemicals where purchased from chemical vendors at reagent grade or higher and used without modification. GC and NMR solvents were of analytical grade, and products used for the enzyme expression were of biological grade.

4.2. Methods. *4.2.1. Enzyme Expression. Section 2.2.1.* The plasmid that encodes Ars-ωTA with its *C*-terminal hexahistidine tag, was transformed into *E. coli* BL21 (DE3) (Novagen from Merck KGaA, Darmstadt, Germany), using standard procedures,[26] and maintained with 100 μg/mL ampicillin. Briefly, Ars-ωTA was expressed in autoinducing medium as follows: a 1% glycerol stock inoculum was used to inoculate 400 mL of ZYP-5052 medium.[27] The culture was incubated at 37 °C, shaking at 250 rpm for 24 h, in a Sartorius Stedim CERTOMAT BS-1. The culture was centrifuged at 8000 rpm (Beckman Coulter Avanti J-26S XP centrifuge, JLA 8.1 rotor) at 4 °C for 30 min, the supernatant were decanted and the cell pellet stored at −20 °C. The average yield was 9 g pellet mass per liter of culture.

Sections 2.1.2, 2.2.2, and 2.2.3. A sample of 1 mL of *E. coli* BL21 (DE3) expressing Ars-ωTA enzyme was inoculated in 50 mL vegetable peptone broth with 20 g/L glucose and 15 μg/mL kanamycin and cultivated for 6−7 h at 37 °C, 250 rpm in a rotary shaker incubator. This preculture was used to seed two 1 L fermentation vessels. Both fermenters were run with the same fermentation procedure, which consisted of culturing the cells in a sugar free semidefined base medium, controlled at pH 7.2, 30 °C, 20% dissolved oxygen, and feeding at a predefined linear rate with base medium containing 400 g/L glucose from the point of inoculation. The culture was induced when 100 OD_{600} was reached by the addition of 0.5 mM IPTG final concentration and reduction in culture temperature to 25 °C. Following 24 h elapsed fermentation time the feed rate was reduced to a predefined constant rate until cell harvest at 41 h. The final cell population reached 212 and 192 OD_{600}, 80 and 81 g/L dry cell weight, respectively. A portion of 1 L of fermentation broth from each reactor was harvested by centrifugation at 6000 g for 35 min; the supernatant was discarded, and the pellet was frozen at −80 °C.

Enzyme Purification. *E. coli* cells (25 g) expressing Ars-ωTA were added to 250 mL 0.1 M phosphate buffer (pH 7) and sonication at 2 °C. The lysate was concentrated by ammonium sulfate to 50−60% ammonium sulfate fraction. The precipitate was resuspended in 25 mL 0.1 M phosphate buffer with 30 mM imidazole, and purified using a His-Trap (Ni-NTA), Ars-ωTA was eluted with 500 mM imidazole. The purified enzyme solution was exchanged into phosphate buffer (0.1 M, pH 7) using an Amicon Ultra 15 Centrifugal filter (10 k) unit and SDS-PAGE analysis performed to confirm that the Ars-ωTA had been purified (>85%) and concentrated (35 mg/mL).

4.2.2. Reaction Conditions. Sections 2.1.1 and 2.1.3. Each reaction, performed in duplicate, contained: 1 g L^{-1} ATA-47, 2 mM PLP, 5% DMSO, 0.1 M tris-HCl buffer pH 7.5, and up to 10 mM pro-chiral ketone acceptor **2** or 1.5 mM pro-chiral ketone acceptor **1** together with 10 mM amino donor **4** or 100 mM **3**, and was run for up to 48 h in 4 mL reaction vessels, at 30 °C in a thermos-shaker. K_{eq} values were determined by measuring conversion at varying concentrations of substrates and products according to a previously described protocol.[20] Inhibition studies were made by measuring initial rates (less than 10% of the limiting substrate consumed), in the presence of increasing product.

Section 2.2.1. All samples were carried out in 0.5 mL reactions in a 96 well plate format. Short reactions of 30 min, 1 h, and 2 h were run with 20 g/L lyophilized cells of Ars-ωTA; the 18 h reactions were run with 2 g/L lyophilized cells. All reactions were run with 10 g/L acceptor **2** in 0.1 M Tris-HCl buffer. The pH optimum was tested at pH's 7, 7.5, 8, and 9, with 0.5 M donor **3**. Donor optimization was tested with concentrations of donor **3** of 0.1, 0.5, 1, and 2 M, carried out at pH 7.5. All experiments were carried out in triplicate.

Section 2.2.2. Reactions were done at 1 mL scale in a 96 well plate, with 11 identical reactions per substrate concentration. The reaction contained 0.1 M Tris-HCl buffer pH 9.0 with 0.4 g/L purified Ars-ωTA, 0.5 M donor **3**, 0.1 g/L PLP, 5−10% DMSO, and 25, 50, or 75 g/L pro-chiral acceptor **2**. The reaction was agitated 250 rpm at 25 °C. Samples were taken at regular time points throughout the experiment.

Section 2.2.3. Reactions were performed at 25 mL scale in an Easymax vessel, which was stirred at 400 rpm, maintained at 25 °C, with substrate concentrations of 25 and 50 g/L. Otherwise the composition in the reactor was identical to that described in section 2.2.2.

Section: 2.1.2. Experiments were carried out by resuspending 500 mg wet cells in 4.75 mL of 500 mM **3** hydrochloride, 50 mM potassium phosphate buffer pH 7.0, and shaking in an orbital shaker at 250 rpm, maintained at 30 °C, for 30 min. 0.25 mL of a 200 g/L solution of pro-chiral ketone acceptor **2** in DMSO was added and the reaction returned to the shaker for 18 h. Reactions were analyzed by GC. The activity for ATA-47 was extrapolated from rates measured in the experiments carried out as described in sections 2.1.1 and 2.1.3.

4.2.3. Product Isolation. Section 2.2.3. The pH of the reaction was adjusted to 13 with 5 M NaOH and extracted with MTBE (3 × 20 mL). The combined organic extracts were filtered through Celite to remove emulsion and dried ($MgSO_4$), filtered, and concentrated in vacuo. Being volatile, the excess amine donor **3** was removed with the organic solvents during concentration.

4.2.4. Work up of Samples for Analysis. Sections 2.1.1 and 2.1.3. The analytical samples were prepared as follows; 0.1 mL of sample was added to 0.4 mL of 1 M NaOH with 10 mM dibenzyl ether as external standard. The compounds were extracted with 0.3 mL of MTBE, and the organic layer was dried with anhydrous $MgSO_4$, which was removed by centrifugation.

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Org. Process Res. Dev. XXXX, XXX, XXX−XXX

Section 2.2.1. The sample was mixed for indicated time at 700 rpm, 30 °C. Samples were sacrificed by addition of 0.5 mL MeCN and spun down. 0.2 mL of the supernatant was transferred to a new plate with 0.8 mL of MeCN and $MgSO_4$. Finally 0.5 mL was transferred to an analysis plate and derivatized with 15 μL of Et_3N and 10 μL of Ac_2O, preceding analysis.

Sections 2.1.2 and 2.2.2. A 1 mL reaction was mixed thoroughly with 9.0 mL MeCN containing 4.5 mg/mL dibenzyl ether. 1.0 mL of this mixture was put in a GC vial and derivatized with 30 μL of Et_3N/20 μL of Ac_2O prior to GC analysis (Chiraldex Dex-CB column 25 m × 0.25 mm × 0.25 μm, oven temp 170 °C for 15 min, Carrier He @ 20 psi, injector/detector 200 °C). Quenched 50 g/L and 75 g/L reactions were further diluted 1:1 with MeCN prior to derivatization and analysis.

Section 2.2.3. 100 μL of the reaction was removed and diluted with MeCN (900 μL). This mixture was derivatized with 30 μL of Et_3N/20 μL of Ac_2O and analyzed by GC.

4.2.5. Analytical. Sections 2.1.1 and 2.1.3. All analytical work was carried out with gas chromatography, with a PerkinElmer (Santa Clara, CA, USA) Clarus 500 apparatus, with PerkinElmer Elite-5 column. 1 μL was injected with a 30:1 split ratio and ran with a constant flow rate of 1.6 mL min^{-1} helium with a temperature gradient from at 120 to 230 °C.

Sections 2.1.2, 2.2.1, 2.2.2, and 2.2.3. Chiraldex Dex-CB column 25 m × 0.25 mm × 0.25 μm, oven temp. 170 °C for 15 min, carrier He @ 20 psi, injector/detector 200 °C.

4.3. ^{1}H NMR of Isolated Product. *N*-Boc-3-amino-piperidine ^{1}H NMR (400 MHz, CD_3OD) 3.96 (1H, m), 3.82 (1H, m), 2.78 (1H, br), 2.67 (1H, m), 2.60 (1H, br s), 1.91 (1H, m), 1.67 (1H, m), 1.44 (9H, s), 1.40 (1H, m), 1.26 (1H, m).

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Notes

The authors declare no competing financial interest.

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Org. Process Res. Dev. XXXX, XXX, XXX−XXX