Development of an integrated chromatographic system for ω-transaminase-IMER characterization useful for flow-chemistry applications

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An integrated chromatographic system was developed to rapidly investigate the biocatalytic properties of ω-transaminases useful for the synthesis of chiral amines. ATA-117, an (*R*)-selective ω-transaminase was selected as a proof of concept. The enzyme was purified and covalently immobilized on an epoxy monolithic silica support to create an immobilized enzyme reactor (IMER). Reactor efficiency was evaluated in the conversion of a model substrate. The IMER was coupled through a switching valve to an achiral analytical column for separation and quantitation of the transamination products. The best conditions of the transaminase-catalyzed bioconversion were optimized by a design of experiments (DoE) approach. The production of (*R*)-1-(4-methoxyphenyl)propan-2-amine and (*R*)-1-methyl-3-phenylpropylamine, intermediates for the synthesis of the bronchodilator formoterol and the antihypertensive dilevalol respectively, was achieved in the presence of different amino donors. The enantiomeric excess (ee) was determined off-line by developing a derivatization procedure using Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide reagent. The most satisfactory conversion yields were 60% for (*R*)-1-(4-methoxyphenyl)propan-2-amine and 29% for (*R*)-1-methyl-3-phenylpropylamine, using isopropylamine as amino donor. The enantiomeric excess of the reactions were 84%$_R$ and 99%$_R$, respectively.

1. Introduction

Enzymes are increasingly used as biocatalysts for the production of fine chemicals and pharmaceutical products [1-3]. In industrial processes, the enzyme should be easily separated from the product and have acceptable stability in order to be reused. These needs can be accomplished *via* immobilization of the enzyme onto a solid support that can be used in batch but also under continuous flow conditions.

Flow reactors can be used for different scopes: analytical scale synthesis (*e.g.* for screening purposes and rapid optimization of the reaction conditions), lab scale synthesis (*e.g.* for pilot studies) or large scale synthesis (*e.g.*, industrial production of active pharmaceutical ingredients, APIs). Biocatalyzed reactions performed in a flow system can benefit from improved mass transfer, excellent temperature control and, importantly, continuous substrate feed and product removal, thus limiting the possible substrate/product inhibition of enzyme activity [4,5].

The implementation of new biocatalytic processes can be a challenging procedure, which may require several stages of characterization and evaluation prior to scale up [6].

In recent years, analytical IMER-based platforms have found several applications in drug discovery with a special emphasis on enzyme inhibitor screening and on-line digestion for proteins characterization [7-10]. However, the potentiality of IMERs for substrate screening and reaction optimization studies on analytical scale, as prototypes for biosynthetic purposes, has not been fully exploited [11,12].

Stereoselective biotransformations are among the different classes of reactions that have been investigated using continuous flow technology. In particular, asymmetric catalysis, has come to the forefront as a highly economical and efficient strategy for the generation of chiral compounds, whereby achiral starting materials are transformed directly into enantio-enriched products [13-15].

It has been recognized that ω-transaminases (ω-TAs) represent an attractive option for the synthesis of chiral amines, which are valuable building blocks for the preparation of APIs that span a range of therapeutic areas [14,16]. Transaminases are pyridoxal 5'-phosphate (PLP)-dependent enzymes that transfer an amino group between an amino donor (*e.g.*, D- or L-alanine, isopropylamine) and a prochiral ketone substrate, thereby creating the C–N bond as well as establishing the key stereogenic center in a single step. At present, more than 60 (*S*)- and (*R*)-selective ω-TAs have been identified, mainly from bacterial and fungal strains [17].

However, some challenges inherent to transaminase-catalyzed processes, such as reaction equilibrium and stereoselectivity, still remain to be tackled before developing large-scale processes [18].

In this context, the use ω-TAs-based IMERs in a continuous flow mode can be useful for the production of chiral amines; two examples of such applications have been reported [19, 20]. In Ref. 19, *E. coli* cells containing overexpressed (R)-selective ω-transaminase and the cofactor PLP, were immobilized on methacrylate beads and used in the asymmetric synthesis of some chiral amines under flow conditions combining a catch and release strategy. Differently in Ref. 20, a homemade monolithic silica support was prepared in a heat-shrinkable Teflon tube and used for the immobilization of transaminase. The reactor was used for the in-flow kinetic resolution of a chiral amine.

In this work, we developed a reliable analytical approach for substrate screening and reaction optimization studies, using a new bioreactor based on (*R*)-selective ω-transaminase from *Arthrobacter* sp., ATA-117 [17,21].

For enzyme immobilization a wide-pore epoxy silica monolithic support was selected. Silica support was selected because of its hydrophilicity to avoid aspecific adsorption of substrates and products. Regarding the morphology, a monolithic material was preferred. Silica monoliths are produced as a single piece of porous material and are characterized by a highly interconnected channel network

In fact, monolithic supports endowed with macropores, guarantee better substrate accessibility due to lack of diffusion resistance during mass transfer, which results in fast enzymatic conversion. These unique properties make them very attractive for bioconversion processes [22,23]. Moreover, these supports are available with different functionalities thus different immobilization chemistries can be used.
For reaction monitoring over time, the bioreactor was inserted in a bidimensional chromatographic system where the reaction mixture can be directly transferred to the analytical column for separation and direct quantification of the product and unreacted substrate. The activity of the bioreactor was determined by setting an on-column enzymatic assay using a model transamination reaction (production of acetophenone).
The best conditions of the transaminase-catalyzed bioconversion were optimized by a design of experiments (DoE) approach and were applied to the synthesis of two chiral amines, (*R*)-1-(4-methoxyphenyl)propan-2-amine and (*R*)-1-methyl-3-phenylpropylamine, useful as intermediates for the synthesis of the bronchodilator formoterol and the antihypertensive dilevalol, respectively. The enantiomeric excess of the reactions was checked by HPLC with a method developed on purpose.
To improve the conversion, diverse strategies were considered including the use of different amino donors.
This work underlines the contribution that the proposed analytical platform can give to enable fast and reliable definition of the best reaction conditions for the manufacture of chiral amines interesting as pharmaceutical building blocks using immobilized enzymes in flow chemistry reactions.

2. Experimental part

2.1. Chemicals

ATA-117 (*R*)-amine transaminase from *Arthrobacter* sp. KNK168, (GenBank: BAK39753.1) was overexpressed and purified as detailed in paragraph 2.2. The enzyme was immobilized on an epoxy-modified silica Chromolith® Flash wide-pore monolithic support (4.6 × 50 mm) (Merck KGaA, Darmstadt, Germany).
Glycine, (*R*)-α-methylbenzylamine (MBA), sodium pyruvate, pyridoxal 5'-phosphate (PLP) hydrate, acetophenone, 4-methoxyphenylacetone (4-MFA), D-alanine, 1-(4-methoxyphenyl)propan-2-amine (4-MPA), 4-phenyl-2-butanone, 1-methyl-3-phenylpropylamine (MPA), Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent), pyruvate decarboxylase, isopropylamine, acetonitrile, methanol and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis,MO, USA).
Potassium dihydrogen phosphate and sodium acetate were purchased from Carlo Erba Reagents (Cornaredo, Italy), while ammonium sulfate and ammonium chloride were from Merck KGaA (Darmstadt, Germany). Water was obtained from a Milli-Q® Integral purification system (Merck KGaA, Darmstadt, Germany).

2.2. Cloning and overexpression of the ATA-117 gene in E. coli BL21(DE3)

The codon-optimized ATA117 gene was synthesized and cloned into the pUC57 vector obtaining pUC-ATA117opt by BaseClear (Leiden, The Netherlands). The ATA117 gene was then amplified using pUC-ATA117opt as a template and primers ATA117fw (GAAGGAGATATACATATGGCGTTCAGTGCCGATACC)/ATA117rv (GTGATGGTGGTGATGATGATACTGCACCGGGGTTAAAAG) that include 18 nt of overlap with the ends

of the pETite vector (Lucigen,WI,USA) for the subsequent cloning into the pETite plasmid in frame with an 6xHis tag sequence. The PCR amplification was carried out on 50 µL reaction mixtures containing 10 ng of plasmid DNA, dNTPs (0.2 mM each), primers (1 µM each), 4 U of XtraTaq Pol and 5 µL of the XtraTaq buffer (both from Genespin, Italy). The PCR conditions were as follows: 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and then 72 °C for 10 min. PCR products were cloned into the pETite vector and transformed into the *E. coli* Hi control 10G using the Expresso T7 Cloning and Expression kit from Lucigen. The resulting plasmid pETiteATA117opt was purified and transformed into *E. coli* BL21(DE3). The obtained transformants were grown in LB supplemented with 30 µg/mL kanamycin (LBkan$_{30}$) medium (50 mL) overnight and then inoculated in 0.5 L LB kan$_{30}$ at 37 °C and 220 rpm. When the OD_{600} reached 0.6–1, gene expression was induced by the addition of 1 mL of 0.5 M IPTG solution in water and the culture was shifted to 30 °C and 220 rpm and grown for further 16 h. Then cells were harvested by centrifugation (5000 rpm for 30 min), resuspended in 20 mL of wash buffer and disrupted by sonication. Wash buffer consisted of potassium phosphate buffer (pH 7.0; 20 mM), NaCl (500 mM) and imidazole (20 mM). For protein purification, clear cell lysates containing soluble protein were subsequently incubated with Nickel Sepharose 6 Fast Flow agarose resin (Ni-NTA) (GE Healthcare, Italy) for 1 h at 4 °C and the mixture was then loaded onto a glass column (10 × 110 mm). The resin was subsequently washed with 10 mL of wash buffer and His-tagged ATA117 was eluted using a 3 step gradient (10 mL washing buffer containing 100, 200, and 300 mM imidazole, respectively) and dialyzed against potassium phosphate buffer (pH 8; 50 mM), at 4 °C. The protein content was measured using the Bio-Rad Protein Assay according to the Bradford method and the protein purity was verified by SDS-PAGE analysis (12% T, 2.6% C). Transaminase activity was assayed by spectrophotometrically measuring the formation of acetophenone at 245 nm ($\varepsilon = 3.66\ mM^{-1}\ cm^{-1}$) [24] and 20 °C on a Jasco V-530 UV/VIS spectrophotometer (Easton, MD, USA) after adding 10-50 µL of purified transaminase to the enzyme assay solution, which consisted of sodium pyruvate (2.5 mM), (*R*)-(−)-α-methylbenzylamine (2.5 mM) in potassium phosphate buffer (pH 8.0 or pH 7.0; 50 mM) and DMSO (0.25%, v/v) in 0.5 mL total volume. One unit of activity is defined as the enzyme activity that produces 1 µmol of acetophenone per minute under the assay conditions described above.

2.3. Immobilization of ATA-117 on monolithic support

Enzyme immobilization was carried out following an *in situ* procedure [23] by flushing in a recycling system an ATA-117 solution through the monolithic support using a HPLC quaternary pump. Before flushing the enzyme solution, the epoxy monolithic column was conditioned with phosphate buffer (pH 7.5; 50 mM) at 0.3 mL/min for 20 min.

The enzyme solution was prepared by diluting the ATA-117 solution (11.68 mg/mL) in ammonium sulfate (1.875 M) in phosphate buffer (pH 7.5; 50 mM) to a final concentration of 2.34 mg/mL (10 mL). During the immobilization procedure, the column was back flushed every 20 min and the flow-rate was maintained constant (0.3 mL/min) for 24 h. The back-flushing procedure was used during the *in-situ* immobilization step in order to obtain an homogeneous enzyme density in the column. Then, an end-capping process was performed by flushing through the support 10 mL of a glycine solution (1 M) in phosphate buffer (pH 7.5; 50 mM). Therefore, the gained bioreactor was evaluated in terms of immobilization yield and catalytic activity**.**

In order to assess the amount of immobilized enzyme onto the monolithic support, a spectrophotometric assay was carried out at 280 nm. An ATA-117 calibration curve was derived (calibration range 0.0365 mg/mL - 0.584 mg/mL). The curve ($y = 0.7512x – 0.0125$) showed a good linearity as expressed by $R^2 = 0.9986$. Therefore, the spectrophotometric assay was carried out on

the enzyme solution applied during immobilization procedure before and after the 24 h. The amount of the immobilized enzyme was estimated by the difference between the two enzyme concentrations, leading to an immobilization yield of 83.1% corresponding to approximately 19.4 mg of enzyme. The amount of immobilized enzyme per unit column volume was 23.42 mg/ml. When not in use, the ATA-117-IMER was stored at 4 °C in phosphate buffer (pH 7.5; 50 mM).

2.4. Chromatography

2.4.1. Apparatus

Chromatographic experiments were performed with three modular systems. *System 1* and *System 2* were used for assembling the column-switching set-up reported in Fig. 1. *System 1* consisted of an Agilent HP-1100 (Palo Alto, CA, USA) pump (pump 1) and a HP-1100 thermostat, delivering the reaction mixture. Pump 1 was directly connected to the bioreactor (ATA-117-IMER) and to a 6-way switching valve (V) containing a 20 µL loop. *System 2* consisted of an Agilent HP-1100 (Palo Alto, CA, USA) pump (pump 2) connected through V to the chromatographic column (LiChospher® 100 RP18, 250 x 4.0 mm, 5 µm). The systems were controlled by an HPLC ChemStation (Revision A.04.01). In this integrated system, the reaction mixture is re-circulated through the bioreactor (System 1, V in position a). After switching V into position b, the analytes (products and unreacted substrates) loaded in the 20 µL loop are directly pumped by System 2 to the analytical column for reaction monitoring. *System 3* was used for ee determination and consisted of an Agilent HP-1100 system (Palo Alto, CA, USA) with a Rheodyne sample valve (20 µL loop) equipped with a HP-1100 diode-array detector and a Zorbax Eclipse XDB C18-Agilent analytical column (150 x 4.6 mm I.D., 5 µm). The chromatographic experiments were carried out at the Department of Public Health, Experimental and Forensic Medicine of the University of Pavia (Italy).

2.4.2. Chromatographic methods

2.4.2.1. In-flow activity assay

The catalytic activity of the immobilized ATA-117 was evaluated by an in-flow activity assay based on the standard reaction reported in Fig. 2A. A solution (9 mL) consisting of (*R*)-α-methylbenzylamine (MBA, 10 mM), sodium pyruvate (10 mM) and pyridoxal 5'-phosphate (PLP, 0.1 mM) in phosphate buffer (pH 7; 50 mM) was prepared and flushed at 0.5 mL/min through the IMER as shown in Fig. 1. For reaction monitoring, 20 µL reaction mixture was diverted to the analytical column. The chromatographic conditions applied for reaction monitoring consisted of isocratic elution using water containing TFA (0.1%, v/v)-acetonitrile containing TFA (0.1%, v/v) (50:50, v/v). The flow-rate was 1 mL/min and the detector was set at 254 nm detection. The stationary phase selected was a LiChrospher® 100 RP18, 250 x 4.0 mm, 5 µm (Agilent Technologies, Palo Alto, CA, USA). The enzyme activity was evaluated by the formation of acetophenone over time.

2.4.2.2. In-flow synthesis of (R)-1-(4-methoxyphenyl)propan-2-amine and (R)-1-methyl-3-phenylpropylamine

In order to synthetize (*R*)-1-(4-methoxyphenyl)propan-2-amine (4-MPA) the reaction considered was the transamination of 4-methoxyphenylacetone (4-MFA) by using D-alanine as amino donor and PLP as enzymatic co-factor (Fig. 2B). The synthesis of 4-MPA was carried out by recycling the reaction solution through the bioreactor. The reaction solution was prepared by mixing D-alanine

as amino donor, 4-MFA as amino acceptor and PLP in different concentrations. The chromatographic conditions for reaction monitoring consisted in an isocratic elution of a binary mobile phase water containing TFA (0.1%, v/v)-acetonitrile containing TFA (0.1%, v/v) (50:50, v/v). The flow-rate was 0.7 mL/min and the detection was set at 225 nm. The stationary phase selected was a LiChrospher® 100 RP18, 250 x 4.0 mm, 5 µm (Agilent Technologies, Palo Alto, CA, USA). The enzyme activity was evaluated by the formation of 4-MPA over time.
The synthesis of (*R*)-1-methyl-3-phenylpropylamine (MPA) was carried out by the transamination of 4-phenyl-2-butanone (Fig. 2C). The reaction mixture was prepared by mixing D-alanine as amino donor, 4-phenyl-2-butanone as amino acceptor and PLP in different concentrations. In this case, the chromatographic conditions for monitoring the synthesis process consisted in an isocratic elution of a binary mobile phase water containing TFA (0.1%, v/v)-acetonitrile containing TFA (0.1%, v/v) (50:50, v/v) at 1 mL/min as flow rate and 254 nm for the detection. The stationary phase selected was a Zorbax Eclipse XDB C18-Agilent (150 x 4.6 mm I.D., 5 µm, 80 Å pore size). The enzyme activity was evaluated by the formation of MPA over time.

2.4.2.3. Enantiomeric excess determination

In order to establish the enantiomeric excess of the produced chiral amines, an off-line derivatization procedure was set up. Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) was used as derivatization agent. The derivatization reaction was performed by dissolving in a vial 0.4 mg of Marfey's reagent [25] in 800 µL of ammonium buffer (pH 9; 1M). 200 µL of reaction mixture were then added to the Marfey's reagent solution. The mixtures were heated at 55 °C for 1 h. In order to calculate the enantiomeric excess, 20 µL of the solution were injected in System 3. The two diastereoisomers were separated using an isocratic elution of a binary mobile phase acetate buffer (pH 5; 20 mM)-acetonitrile (60-40, v/v). The flow-rate was 0.8 mL/min and the detection was set at 340 nm.

2.5. Calculation

Conversion percentages were calculated as:
C = [product concentration/(product concentration + substrate concentration)]x100
where product and substrate concentrations were calculated from the calibration curves.
The product enantiomeric excess was calculated as follows: $ee_p = (x_1-x_2)/(x_1+x_2)$; where x_1 and x_2 denote the areas of the two diastereoisomers separated on the analytical column.

2.6. Quantitative analysis

For quantitation, three different calibration curves were obtained. For each of the three reaction products (acetophenone, 4-MPA and MPA), a 10 mM stock solution was prepared in phosphate buffer (pH 7; 50 mM) and five dilutions were prepared in the same solvent. Each solution was injected in triplicate. 4-MPA solution was prepared at the Department of Public Health, Experimental and Forensic Medicine of the University of Pavia (Italy).
The chromatographic conditions used for the calibration curves are reported in paragraph 2.4.2. The calibration ranges were 0.05 mM – 10 mM for acetophenone and 0.1 mM – 2.5 mM for 4-MPA and MPA. The lowest level of the calibration curve of acetophenone correspond to 0.5 % conversion, while the lowest level for 4-MPA and MPA corresponds to 1 % conversion. The highest level of the three curves correspond to 100 % conversion.

The calibration curve equation computed for 4-MPA was y = (557±4)x - (11±5), with a correlation coefficient R^2 = 0.9998, demonstrating excellent fitting. The slope is highly significant ($p < 0.0001$) and the intercept is not significant (p=0.1195). The calibration curve equation of MPPA was y = (241.6±0.6)x + (0.4±0.7), with a correlation coefficient R^2 = 0.99998, demonstrating excellent fitting. The slope is highly significant ($p < 0.0001$) and the intercept is not significant (p=0.5450). The calibration curve equation of acetofenone was y = (9053±170)x + (1210±875), with a determination coefficient R^2 = 0.9989, demonstrating satisfactory fitting. The slope is highly significant ($p < 0.0001$) and the intercept is not significant (p=0.2608).
For the calibration curves each standard solution was injected in triplicate. The precision of the methods was estimated calculating the RSD% of the three replicates. In all cases RSD% lower than 3.5% at the lower limit of the linear range was obtained (maximum RSD% 3.2% for 4.MPA, 2.5% for MPPA, and 1.1% for acetofenone).

2.7. Experimental design

The synthesis of 4-MPA catalyzed by ATA-117 (Fig. 3), was used as reference reaction for the experimental design aimed at maximizing the conversion yield. A Plackett-Burman design of experiment was applied. Six process factors and one "Dummy" fictitious variable were considered for evaluating the responses. These were temperature (X_1), pH (X_2), PLP mM concentration (X_3), Ala/Ketone molar ratio (X_4), flow rate (X_5), and buffer molar concentration (X_6). Given the number of factors, a Plackett-Burman design of 8 runs was chosen in order to screen the relevance of each of the six factors selected. In addition to the eight experiments, three supplementary test reactions were conducted under the following conditions corresponding to the center point of the experimental levels, except for the Dummy variable: temperature 35°C (X1 = 0), pH 7.5 (X2 = 0), PLP concentration 1.05 mM (X3 = 0), Ala/Ketone molar ratio 6:1 (X4 = 0), flow rate 0.3 mL/min (X5 = 0), buffer concentration 42.5 mM (X6 = 0), and Dummy variable set at the -1 level, to check method performance as well as to estimate the experimental error (Table 1, Experiments TC, Test Conditions, experiments).
The experiments reported in Table 1 are listed according to the standard order obtained after building the Plackett-Burman experimental plan according to references [26,27]. The percentage of conversion yield at 4 h (Y_1) and 24 h (Y_2) were the responses (Table 1).
The model underlying the Plackett-Burman design is first order in each factor and in the present case is represented by the equation: $Y = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3 + b_4 \cdot X_4 + b_5 \cdot X_5 + b_6 \cdot X_6 + b_7 \cdot X_7$.
It is hypothesized that the interactions between the factors can be completely ignored thus limiting drastically the number of experiments required to compute the main factors effects.
The coefficient b_7 of the Dummy variable (X_7), in all the models computed can be considered a direct measure of the total error (random + systematic) existing in the data collected. The data collected were studied using Microsoft Excel 2010 and R for Microsoft Windows, version 3.2.3, Copyright(C) 2014, R Foundation for Statistical Computing. R-based chemometric software routines were used for DoE calculations. The R-based software has been developed by the Group of Chemometrics of the Italian Chemical Society [http://gruppochemiometria.it/gruppo-lavoro-r-inchemiometria.html].

3. Results and discussion

In this work, the development of an IMER based on an (*R*)-selective ω-transaminase (ATA117) for reaction optimization on analytical scale, is described. The most appealing benefits offered by analytical IMERs technology are the low sample consumption and the possibility of connecting the reactors to different separation and detection systems. In addition, the high enzyme-to-substrate

ratio, achievable with these systems, can significantly improve reaction efficiency and shorten the reaction times required to rapidly screen different reaction conditions/substrate specificity in view of preparative applications.

3.1. Bioreactor preparation

IMERs can be prepared in various formats using different support materials and re-used for multiple cycles.
In this study, a wide-pore epoxy silica monolithic support was selected for the covalent immobilization of the enzyme following an *in situ* procedure. This support exhibits micrometer size flow-through pores, thus forming a macroporous network, and nanometer size mesopores on the skeleton that result in fast kinetics and high binding properties [22]. Silica monolith is a hydrophilic material, an important characteristic to avoid substrate adsorption. In addition, when used as enzyme carrier, this material can ensure an easier accessibility of substrates to the active site as a result of the absence of diffusional limitations typically encountered with conventional silica particle supports [22,23].
ATA-117 enzyme was overexpressed in *E. coli* cells and purified to homogeneity by affinity chromatography. The recovery yield was 177 mg of pure protein from 0.5 L-culture (15 mL, 11.8 mg/mL). The transaminase activity of the purified enzyme, under the previously described conditions, was 4.21 U/mL and 11.53 U/mL at pH 7.0 and pH 8.0, respectively. Purified ATA-117 was immobilized on epoxy-activated monolithic silica following the immobilization protocol described in paragraph 2.3. The amount of immobilized enzyme, as estimated spectrophotometrically, was 19.4 mg corresponding to an immobilization yield of 83%. The amount of immobilized enzyme per unit column volume was 23.42 mg/ml.

3.2. Bioreactor characterization and stability

Evaluation of the enzyme activity after immobilization was the next step. The activity of the ATA-117-IMER was assayed by submitting the bioreactor to an in-flow activity assay (production of acetophenone, see Fig. 2A) based on the conversion of the model substrate (*R*)-α-methylbenzylamine and using the column switching set-up (Fig. 1). The experimental conditions are detailed in paragraph 2.4.2.1. The reaction mixture was prepared as described in paragraph 2.4.2.1, and the product of this reaction, acetophenone, was quantified over time by an HPLC method developed on purpose. A typical chromatogram is reported in Fig. 3A. A conversion percentage of approximately 90% was obtained after 21 h allowing the equilibrium conditions (Fig. 3B). The initial rate of the reaction was estimated from the slope of the curve at the beginning of the reaction, from 0-4 h (Fig 3B). The in-flow initial rate of product formation over time was 0.144 µmol/min (MBA, 10 mM and sodium pyruvate 10 mM). In batch conditions, considering the specific activity of purified transaminase at pH 7.0 (4.21 U/mL), it can be estimated a 0.2105 µmol/min conversion rate.
Although a direct comparison with the same biotransformation carried out in batch conditions is not feasible, due to the different experimental conditions used (*i.e.* enzyme amount, assay configuration etc.), these results highlight the good performance of the in-flow approach.
This assay was also used to test the stability of the bioreactor over time. ATA-117-IMER has retained the initial catalytic activity over 35 reactions in 4 months. A variation percentage of less than ± 5 % has been considered as nonsignificant and linked to the experimental variability. After one year from the bioreactor preparation, the long term stability was evaluated using the same assay and a loss of activity (15%) was measured.

3.3. Bioreactor applications: in-flow synthesis of (R)-1-(4-methoxyphenyl)propan-2-amine (4-MPA) and (R)-1-methyl-3-phenylpropylamine (MPA)

The synthesis of enantio-enriched amines using the ATA-117-IMER can be achieved by asymmetric synthesis starting from prochiral ketones. This is an attractive approach since a theoretically 100% yield can be obtained.

The ATA-117-IMER was used to study the synthesis of 4-MPA, a precursor of the β2 agonist formoterol, from the corresponding ketone (Fig. 2B). As starting amino donor, the commonly used D-alanine was chosen. This stereoselective synthesis was already described using the free enzyme in solution [28].

To assess the reaction conditions, a DoE approach based on Plackett-Burman model was applied. Eight different reactions were carried out to establish the effect of 7 variables (temperature, pH, PLP concentration, amino donor/ketone, flow rate, buffer concentration and one dummy variable) on the reaction rate.

The six parameters, as well as their nominal values, were selected on the basis of theoretical considerations and/or preliminary experiments (Table 1). When an in-flow reaction is considered, flow rate determines the contact time between enzyme and substrate. Two extreme flow rate values (0.1 and 0.5 mL/min) were chosen. When hydrophobic interactions occur in enzyme-substrate binding, high ionic strength can improve enzymatic activity by lowering the Michaelis-Menten constant (Km). Thus, experiments were carried out with 10 and 75 mM buffers. The effect of pH, PLP concentration and T was also included. An excess of amino-donor is required to shift the reaction equilibrium, for this reason two different alanine/ketone ratio were considered.

For each variable, two levels (one higher and the other lower) were applied, and the conditions are listed in Table 1. The experimental error was evaluated by replicating three independent runs in the center point of every process factor with the exception of the Dummy variable.

Plots of magnitude of the coefficients of the Plackett-Burman model, together with the corresponding confidence intervals, are reported in Fig. 4. For coefficient evaluation, a confidence interval was calculated as $(t \cdot S.D.exp)/\sqrt{N}$ where N is the number of experiments of the Plackett-Burman design (eight), S.D.exp the experimental standard deviation calculated from the three TC Experiments, and t the Student variable at 95% confidence level (dotted line).

The data showed that at 24 h the significant parameters were the ratio between amino donor and prochiral ketone, and the flow-rate. The positive effect of the high flow rate on the conversion yield is very interesting and underlines the necessity to remove the reaction product from the catalytic site in order to avoid the inhibition of the enzyme. Factor X_1, the temperature, is certainly important. The model evidences the positive effect of the temperature only at the beginning of the reaction, while at 24 h, the effect is negligible. Cofactor concentration, buffer concentration, and pH could not be distinguished from the noise of a random variation in the yield of the reaction.

The low conversion yield obtained (less than 10%) is not surprising since organic synthesis by ω-transaminases suffers from an unfavorable reaction equilibrium, especially in the asymmetric synthesis of amines from an amino acid as amino donor. In fact, to maximize the productivity of ω-transaminases it is necessary to shift the equilibrium following process control strategies such as the in situ removing of the product, or the use of cascade reactions [17].

Following the results of the DoE approach, the experimental conditions were further modified in order to shift the reaction equilibrium, this can be easily carried out with the chromatographic platform. First, soluble pyruvate decarboxylase (140 μg enzyme) was added to the reaction mixture to remove pyruvate co-product. The advantage of using pyruvate decarboxylase is that it does not require recycling of cofactor and the byproducts formed are highly volatile, therefore they can be

easily removed (Fig. 5A) [28]. However, in the considered conditions, the conversion yield did not improve significantly.
As a second strategy, isopropylamine was considered as alternative amino donor and it was added to the mixture in the same molar ratio as D-alanine (10:1) and increasing the molar ratio (30:1) (Fig. 5B). This amino donor is very interesting because of the simple removal of the acetone co-product. The three reaction mixtures were prepared in phosphate buffer (pH 7; 75 mM), PLP (0.1 mM) and the temperature was set at 50 °C. The experiments were carried out in the recycling system described above at 0.5 mL/min for 24 h. The amine quantitation was monitored by an HPLC method developed on purpose as well as for ee determination. Representative chromatograms of the reaction monitoring and ee determination are presented in Fig. 6A and 6B, respectively. In order to compare the different conditions (Fig.7), all the reactions were monitored at a fixed time (24 h).
A significant increase of the conversion yield was obtained by using isopropylamine as amino donor (20% conversion yield). An increase in the molar ratio between the amino-donor and the prochiral ketone from 1:10 to 1:30 allows to reach a 60% conversion yield in 24 hours.
As far as 4-MPA concerns, 98% conversion has been previously reported using ATA-117 in solution with shaking at 30 °C for 24 h and coupled to a lactate dehydrogenase (LDH)-based system for the shifting of the reaction equilibrium [28].
This result achieved without the use of biochemical cascade reaction is very interesting and could be further improved by applying catch and release strategies in flow chemistry applications.
In these optimized conditions (1:30), the transformation of 4-phenyl-2-butanone at 10 mM substrate concentration yielded (*R*)-1-methyl-3-phenylpropylamine with 29 % conversion after 24 h. For this reaction the repeatability of the conversion yield at 24 h was determined in triplicate in three consecutive days. A mean conversion yield of 25 ± 4% was determined. The lower conversion yield obtained with this product is in agreement with the in batch results reported in the literature [28].
Enantioselective reactions are the result of the competition between different possible diastereomeric reaction pathways, through diastereomeric transition states when the prochiral substrate complexed to the chiral catalyst reacts with the corresponding reagent.
At 24 h the ee was determined for both chiral amines. An enantiomeric purity of 84% ee was obtained for 4-MPA while 99% ee was reached for MPA.
The same reactions were performed in batch conditions. The conditions were: pH 7, 75 mM phosphate buffer, 0.1 mM PLP, T= 50 °C. Isopropyl amine/prochiral ketone ratio (30:1) in 9 ml reaction mixture. 2,336 enzyme were added to the reaction mixture (0,259 mg/ml). In these experiments a maximum conversion yield (approximately 30 and 10 %) was obtained after 24 h for 4-MPA and MPA respectively. The ee was also measured and no significant variation in respect to the in-flow experiments was observed.

4. Conclusions

An integrated platform involving transamination using an IMER based on ATA-117, on-line reaction monitoring and enantiomeric excess determination was established.
This work underlines the contribution that an analytical platform can give to derive the best reaction conditions, achieving interesting conversion yields, for the manufacture of chiral amines as important pharmaceuticals building blocks. The defined optimal conditions for 4-methoxyphenylacetone enantioselective conversion are likely to be applicable to other structural correlated substrates. However, the integrated platform can be easily applied in the optimization of other specific substrates to be converted or to characterize other ω-transaminases with different specificities.

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Figure Captions

Fig. 1. Integrated HPLC set-up comprising the ATA-117-IMER, the analytical column for reaction monitoring and the system for enantiomeric excess (ee) determination.

Fig. 2. (**Panel A**) Reaction of acetophenone production catalyzed by ATA-117; Transamination reactions for the production of (**Panel B**) (*R*)-1-(4-methoxyphenyl)propan-2-amine catalyzed by ATA-117, and (**Panel C**) (*R*)-1-methyl-3-phenylpropylamine catalyzed by ATA-117.

Fig. 3. A. Representative chromatograms for the in-flow activity assay (production of acetophenone). Results of the reaction monitoring at 0 (blue) and 21 h (red) are reported.

B. Monitoring over time of the in-flow activity assay. Calculated concentrations of substrate ((*R*)-α-methylbenzylamine, (*R*)-α-MBA) and product (acetophenone) are reported.

Fig. 4. Plot of magnitude of the coefficients and of the confidence interval (dotted line) of the Plackett-Burman model for the response (Y_1) represented by the conversion yield at 4h **(A)** and (Y_2) represented by the conversion yield at 24h **(B)**. The model equation is $Y_1 = 5.86 + \mathbf{1.37}\cdot X_1 - 0.825\cdot X_2 - 0.248\cdot X_3 + \mathbf{2.06}\cdot X_4 + \mathbf{1.72}\cdot X_5 + 1.06\cdot X_6 - 1.05\cdot X_7$ for **(A)** and $Y_2 = 7.04 + 0.326\cdot X_1 - 0.204\cdot X_2 - 0.936\cdot X_3 + \mathbf{2.32}\cdot X_4 + \mathbf{1.28}\cdot X_5 + \mathbf{1.06}\cdot X_6 - 1.01\cdot X_7$ for **(B)**.

Fig. 5. Strategies to increase the yield of the transamination reaction: (**A**) addition of soluble pyruvate decarboxylase to the reaction mixture; (**B**) use of isopropylamine as alternative amino donor.

Fig. 6. (**A**) Representative chromatograms for the in-flow production of (*R*)-1-(4-methoxyphenyl)propan-2-amine starting from 4-methoxyphenylacetone and isopropylamine in a 1:10 molar ratio. Results of the reaction monitoring at 0 (blue) and 23 h (red) are reported.

(**B**) Representative chromatograms for the enantiomeric excess determination in the production of (*R*)-1-(4-methoxyphenyl)propan-2-amine (4-MPA) starting from 4-methoxyphenylacetone and isopropylamine in a 1:30 molar ratio. Chromatograms of the reaction mixture at 24 h (red) compared to the racemic amine (blue), both after the derivatization procedure using Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent), are presented.

Fig. 7. Conversion yield monitoring over time of the four reaction mixtures for the production of (*R*)-1-(4-methoxyphenyl)propan-2-amine. Ala = alanine, PDC = pyruvate decarboxylase, IPA = isopropylamine.

Table Caption

Table 1. Conditions applied for the 8+3 experiments (Exp) of the Plackett-Burman design and Test Conditions (TC).

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